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TITLE: UG311, An Oncofetal Marker Lost With Prostate Cancer Progression

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13. ABSTRACT *(Maximum 200 Words)*

Purpose: The mRNA expression of a paralogous human sequence to UG311, a murine urogenital sinus expressed sequence tag, was found to decrease with androgen independent progression in the LNCaP model of human prostate cancer progression as determined by RNA blotting. Analysis of the sequence of UG311 determined significant homology to a single-stranded nucleic acid binding protein, nmt55. To exclude nmt55 as the gene corresponding to UG311, antibodies and cDNAs were acquired.
Scope: As members of the single-stranded nucleic acid binding protein family, nmt55 and UG311 may play a role in DNA repair or RNA splicing. Both functions are currently under evaluation for nmt55. Either of these functions is likely to have impact on the progression and potential therapeutic outcome for prostate cancer patients. Improper splicing would lead to entire classes of proteins being disrupted. Loss of DNA repair enzymes would lead to increased genomic instability and accelerated progression.
Major Findings/progress: As outlined in Aim 2, human prostate tissues and cell lines were screened by IHC. RNA blot analysis was performed to compare with the expression of UG311 in the LNCaP progression model. The data indicate that nmt55 expression does not change with stage or grade nor does the expression of nmt55 mRNA resemble the loss of expression previously observed for UG311 in the LNCaP model of prostate cancer progression. Therefore, the goals of aim 1 become more pertinent. To this end current efforts are focusing on the cloning of the UG311 paralog by RACE and phage library screening. A lambda ZAP library has been constructed from C4-2 cells to screen for paralogous clones. RACE assays are underway.

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Introduction

The subject of the current research is to discern whether UG311 and nmt55 are the same gene. Preliminary sequence analysis indicated a large degree of homology over the area of the UG311 expressed sequence tag (EST), however, the RNA size for UG311 is different than that reported for nmt55. Since reagents are available for nmt55 then the pattern of expression will be determined to see if it is the same as UG311. If not, then UG311 is likely to be a novel member of this family and warrants extensive investigation. The purpose is to determine the expression pattern and the association of nmt55 and UG311 in prostate carcinogenesis and progression. Since UG311 is lost in aggressive prostate cancer cell lines, this gene will be re-expressed in aggressive prostate cancer to study its ability to normalize the cancer cell phenotype. The ultimate scope of this work is to develop new prostate cancer biomarkers or therapeutic targets.

Body

Aim 1a. In collaboration with Fan Yeung (graduate student in Urology/Biochemistry) a Lambda-ZAP library has been constructed from C4-2 cells. The advantages of using Lambda-ZAP (Stratagene Inc, La Jolla, CA) are 1) Larger insert size (12kb max) 2) both prokaryotic and eukaryotic expression and 3) rescue to plasmid. C4-2 was chosen to decrease the number of positive colonies upon hybridization with UG311-est sequences. The library has been titered at $>1.1 \times 10^7$ pfu/µl phage. Screening is about to initiate (Aim 1b). Aims 1c-d require the results of aim 1b. **Note Bene**: the use of Lamda-ZAP system also satisfies portions of aim 1g where the insert was to be cloned into separate prokaryotic and eukaryotic expression vectors.

Aim 1e. RACE reactions. As shown in Figure 1 5'- and 3' RACE reactions have been optimized for control reactions. Furthermore, candidate bands have been gel purified, cloned into pCR2.1-TOPO (a PCR product cloning vector). Several of the bands have been pulled, cloned, amplified and sequenced. Sequence alignment with the original UG311 sequence has not been completed at this time. The GenBank comparisons and alignment are part of aim 1f.

Aim 2a. Immunocytochemistry/immunohistochemistry of human cell lines and tissues. The object of this aim is to determine if nmt55 has the same expression pattern as UG311, namely decreased expression with prostate cancer progression. The rationale is two-fold. First, nmt55 protein expression is lost with breast cancer progression and aggressiveness. Second, UG311 has significant homology with nmt55 in the EST overlap and may actually be nmt55. Our data for UG311 are form northern blots since no antibodies exist at this time. Therefore, the initial screen for nmt55 expression was done by RNA blot using a 3'-specific nmt55 cDNA probe (Figure 2A). Contrary to the results for UG311 (Figure 3 of original grant), the mRNA expression of nmt55 does not decrease across the LNCaP progression model. Also, the UG311 mRNA was estimated to be 3.2 kb. This gel indicates that the nmt55 specific probes hybridizes to a 2.5 kb mRNA. Unfortunately, beta-actin appears to be independently regulated and highly variable in these cell lines and, as such, does not serve as a good loading control. This will be redone using GAPDH or beta-2-microglobulin which have served as reliable loading controls in previous RNA blots and PCR reactions and should allow for transcript normalization to the control.

Immunocytochemistry was then performed using a polyclonal antibody (nmt5) to nmt55 acquired from R. Moreland. Prostate cancer cells were fixed on the dish in Zn-buffered formalin and were subsequently scraped from the dish. The fixed cells were pelleted, the formalin removed and the pellets resuspended in 42°C agarose. The agarose was allowed to harden whereupon the pellets were paraffin-embedded and sectioned. Antigen retrieval was performed in sodium citrate buffer with 16 minutes on high (from cold starting solution) plus 15 minutes at 40% power. Primary antibody incubation was at 37°C for 1 hour. All slides were treated simultaneously for antigen retrieval, incubation, washes and color development. As indicated in figure 3 there

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is no appreciable loss of nmt55 staining with the progression model. Nor does there appear to be reduced amounts of nmt55 in the androgen receptor deficient prostate cancer cell lines DU145, PC-3 and PC-3M. These data confirm that nmt55 is most likely not UG311despite the homology of the core sequences. They are probably related family members. Further, these data support the contention that actin is a poor control for the mRNA blots since normalization to actin would have predicted and overall decrease for nmt55 that is not the reality of either the RNA blot or immunohistochemistry.

Immunohistochemistry was performed on human prostate cancer specimens but no difference was found. Nmt5 stained sections were not retrievable from pathology at this time due to scheduling conflict.

Key Research Accomplishments

- Screened of human prostate cancer specimens for expression of nmt55 protein using nmt5 and nmt1 antibodies. No change in protein expression with stage or grade was found. (Aim 2)
- Screened prostate cancer cell lines for expression of nmt55 protein. Cell lines include: the LNCaP prostate cancer progression model (LNCaP, C4-2, C4-2B2, C4-2B3, C4-2B4, C4-2B4(6-1), and C4-2B5)as well as P69 (a T-antigen transformed prostate epithelial cell line), Du145, PC-3 and PC-3M (androgen independent prostate cancer cell lines). 1) no difference was found in expression levels of nmt55 mRNA by Northern blot and 2) no difference was found in expression of nmt55 by immunocytochemistry of concurrently processed cell blocks. (Aim 2)
- C4-2 lambda phage library construction completed in collaboration with Fan Yeung (Ph.D. Candidate in Biochemistry) (Aim 1a)

Reportable Outcomes

- The development of the urogenital sinus library and the screening of 746 clones in the LNCaP progression model. This manuscript is being held up for processing of the patent application for eleven of the UGS-derived sequences that show differential expression, including UG311.
- Patent on UGS-derived oncofetal markers. (Provisional #60/085,383 5/14/98, Patent Conversion initiated June 2000)
- Appendix 2. Gregory CW, Degeorges A and **Sikes RA***. The role of the IGF axis in the development and progression of prostate cancer. In Recent Research Developments in Cancer, (Eds) Pandalai.S.G., Mukhtar H and Labrie F, Transworld Research Network, Kerala, India. March 2001. Volume X, Chapter Y , ppxxxx-xxxx, March 2001 (*In Press)* **Corresponding Author** Since IGFBP's and other IGF-axis members were found to be expressed in the UGS library and may be important in prostate development and prostate cancer progression, this chapter does tie in directly with the goals of this grant. Also, since this occupied a significant portion of my time, then acknowledgement of DOD support is appropriate.

Conclusions

- Since the expression pattern of nmt55 is constant and UG311 decreases with progression of prostate cancer cell lines, then nmt55 and UG311 do not encode the same gene.
- Nmt55 expression in prostate cancer does not correlate with stage or grade. This is contrary to the expression profile observed for nmt55 in breast cancer.
- This increases the likelihood that the UG311 paralog is a novel member of this type of single-stranded nucleic acid binding protein family.

References

None

Legends to Figures

Figure 1. A) RACE control reactions using LNCaP-derived cDNAs. TFR is the transferrin receptor control. Int. is an internal RACE reaction control. B) Sample experimental RACE results. LNCaP cDNAs were subjected to 5'- and 3'-RACE reactions using UG311 specific primers as the anchors. The complexity of the 5'-RACE reaction is indicative of either multiple family members, splice variants, or sub-optimal PCR conditions.

Figure 2. A) Northern Blot analysis of prostate cancer cell lines. Sample order is the same as for 1B. Thirty micrograms of total RNA was separated by denaturing gel electrophoresis followed by electrophoretic transfer to ZetaProbe (BioRad Labs,) Nylon membrane. Membranes were prehybridized at 65 degrees celsius for 3 hours in RapidHyb Buffer (Amersham Life Science, Arlington Heights, IL) containing 1 µg/ml tRNA. Hybridization buffer was changed and 1×10^6 dpm/ml of nmt55 or during reprobe with beta-actin cDNA probes. Hybridization for 16 hours followed by washes to moderate stringency (0.5x standard saline citrate, 1% SDS, 65°C, 2 x 15 minutes). Membranes were placed onto X-ray film for exposure. B) Autoradiograms from A were scanned using a Molecular Dynamics densitometer and the intensity of the bands was calculated and normalized to the values acquired for LNCaP.

Figure 3. Immunocytochemistry of nmt55 in Zn-buffered formalin fixed prostate cancer cell line pellets. A,C,E,G,I,K are hematoxylin and eosin stained while B,D,F,H,J,L are stained with nmt5 polyclonal antibody at 1/4000 dilution in PBS/1%BSA using DAB as chromagen. Key: A,B-LNCaP; C,D-C4-2; E,F-C4-2B4(6-1); G,H-DU145; I,J-PC-3; K,L-PC-3M. Magnification 31.25X final.

Appendices

1. Figures 1, 2, 3.

2. Gregory CW, Degeorges A and **Sikes RA***. The role of the IGF axis in the development and progression of prostate cancer. In Recent Research Developments in Cancer, (Eds) Pandalai.S.G., Mukhtar H and Labrie F, Transworld Research Network, Kerala, India. March 2001. Volume X, Chapter Y , ppxxxx-xxxx, March 2001 *(In Press)** **Corresponding Author**

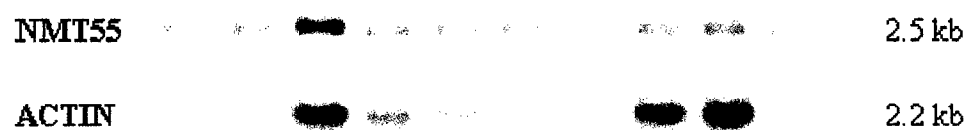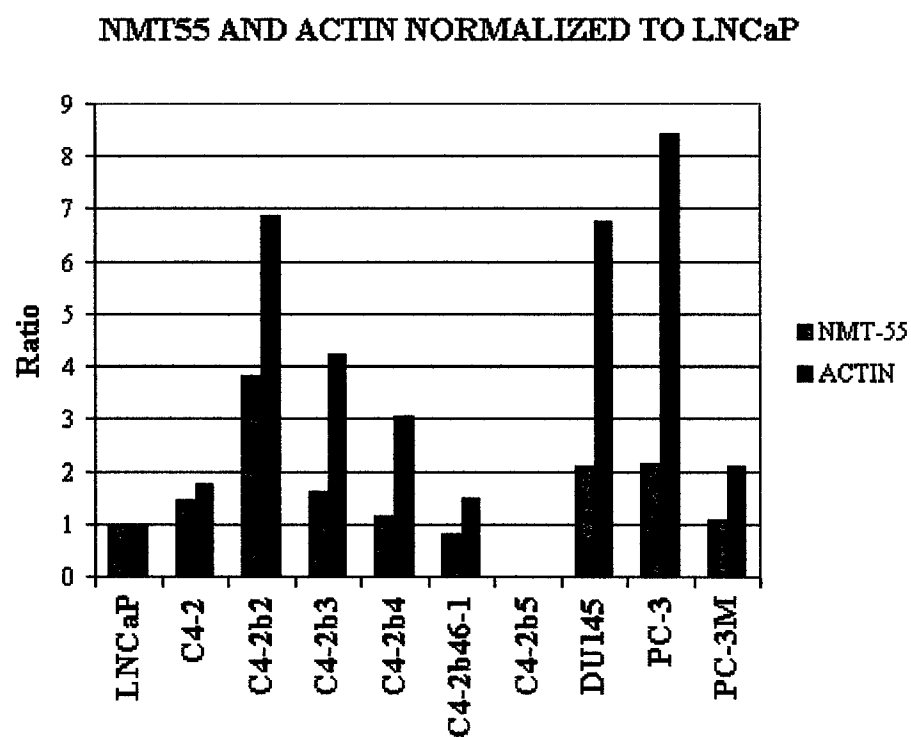3. Curriculum Vitae: Robert A. Sikes, Ph.D.

A.

B.

FIGURE 1

A.

NMT55   2.5 kb

ACTIN   2.2 kb

B.

NMT55 AND ACTIN NORMALIZED TO LNCaP
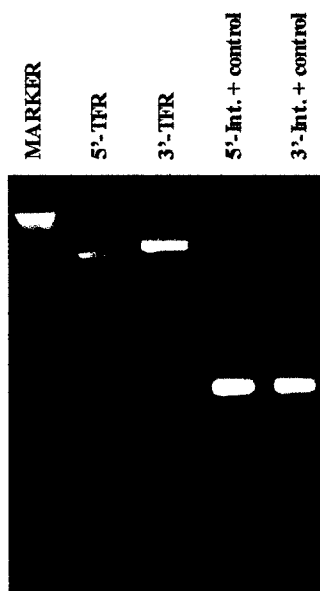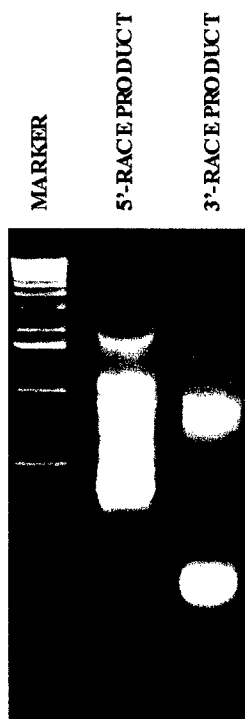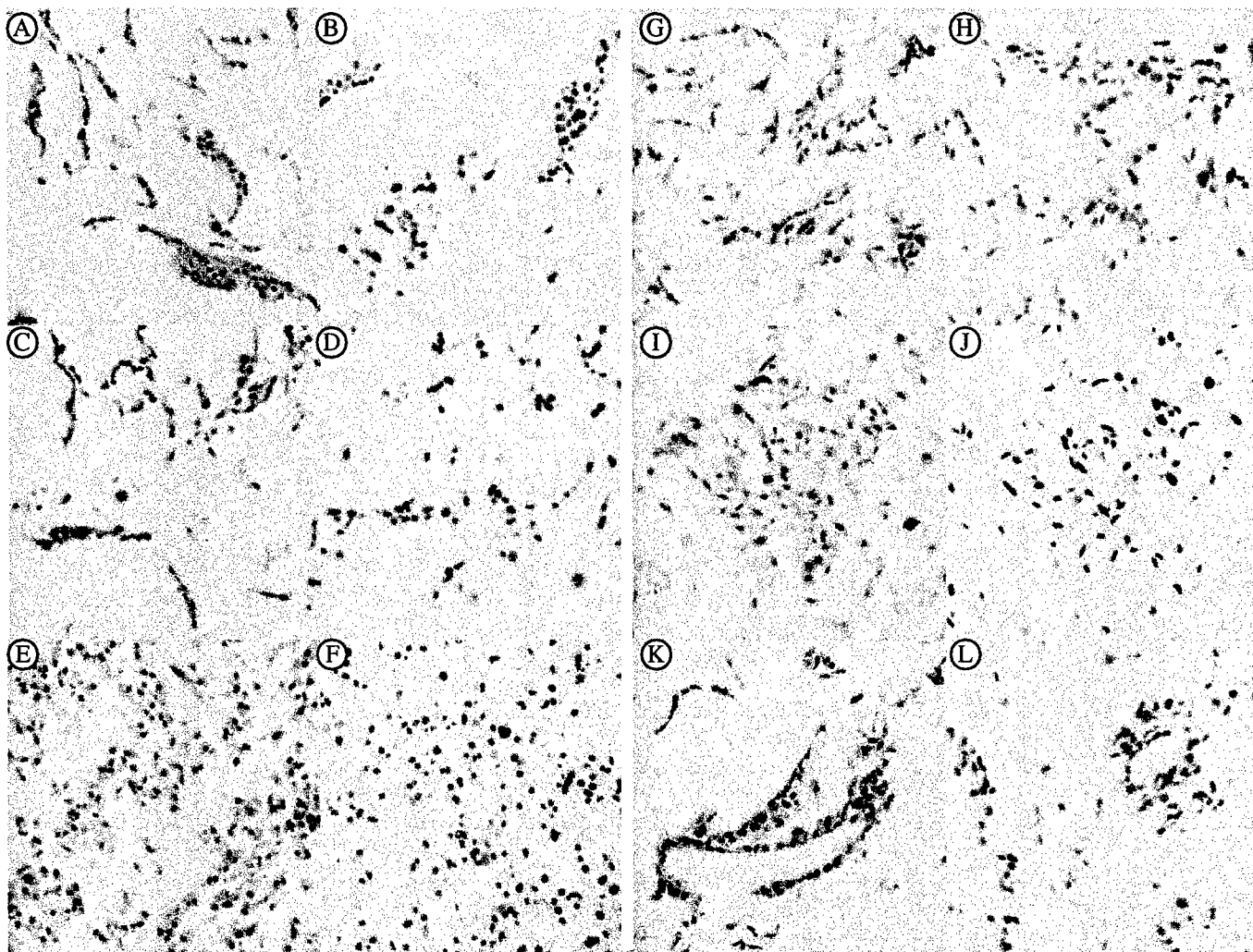
FIGURE 3

THE IGF AXIS IN THE DEVELOPMENT AND PROGRESSION OF PROSTATE CANCER.

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Running title: IGF axis in prostate neoplasia.

I. ABSTRACT

Worldwide, prostate cancer is the third most commonly diagnosed cancer in men and the sixth most common cancer related mortality [1]. In North America prostate cancer is the most frequently diagnosed cancer and remains second only to lung cancer as the leading cause of cancer mortality [2]. The overall lifetime risk of developing an invasive prostate cancer is 1 in 6 for males in North America. With an estimated 396,000 cases worldwide and a 41.6% mortality rate, the impact of prostate cancer on the resources of health care is dramatic. Despite increased awareness and the widespread use in North America of the prostate-specific antigen (PSA) blood test, the incidence and mortality rates for prostate cancer have not changed significantly. Therefore, prostate cancer research strives to understand the mechanism(s) behind the development and progression of prostate cancer. As prostate cancer grows and progresses from localized to metastatic disease, there are changes in cellular adhesion and extracellular matrix molecules [3-10], motility [7-9, 11], responsiveness to androgenic steroids [6, 12-14], as well as dysregulated expression and/or response to peptide growth factors (GFs) or by GF receptors [15-17]. A better understanding of the changes in these systems will provide the basis for the design of new and more effective therapies to treat prostate cancer. The goal of this chapter is to provide an update on the role of the insulin-like growth factor axis in the development and progression of prostate cancer.

II. INTRODUCTION

Overview

The insulin-like growth factor (IGF) axis is comprised of two ligands (IGF-1 and IGF-2), the insulin-like growth factor receptors type I and type II (IGF1R and IGF2R, respectively), and six high affinity IGF binding proteins (IGFBP-1 to -6) [18-20]. On the surface, this appears to be a simple and straightforward system. The reality, however, is quite different. The action of the IGFs on various target tissues is mediated through systemic endocrine control of IGF-1 production by the liver. Target tissues also produce their own IGF-1 and -2. Furthermore, the effects of IGF in target tissues depend upon the availability and number of IGF receptors as well as the local production of the IGFBPs, which often varies by number and type within each target tissue. Ultimately, the net effect of the IGFs is determined by the interaction of the IGFBPs with the extracellular matrix and by the local production and action of IGFBP proteases.

Ligands

The ligands for the IGF axis are IGF-1 and IGF-2, which are 67 and 70 amino acids, respectively. They play a role in several critical biologic processes: apoptosis, cell cycle progression, differentiation, and regulation of gene expression [19]. IGF2 is important generally for fetal development whereas IGF-1 is dominant after birth. IGF is thought to mediate most of the effects of growth hormone [21-23]. This being said, rodents downregulate IGF-2 expression in adults while humans continue to express both ligands through adulthood [24, 25].

The crucial role for the IGF axis in development was demonstrated by genetic knockout of different members of the IGF axis, either alone or in combination, in transgenic mice [23, 26-28]. Single deletions of either IGF-1 or IGF-2 resulted in a marked reduction in postnatal survival and growth deficiency of offspring to only 60% of control. Double knockouts resulted in neonatal lethality and further exacerbation of the dwarfism to about 30% of control size [28]. By contrast, the overexpression of GH or IGF-1 in transgenic animals resulted in dramatic overgrowth of somatic tissues, but these studies also demonstrate that GH and IGF-1 have independent actions [29-32]. With the exception of the testes, kidneys, and adrenal glands, IGF-2 overexpression did not result in similar overgrowth of somatic tissues in transgenic animals [33]. The binding affinities of IGF-1 and IGF-2 to the IGF1R are quite similar at 1 nM and 3 nM, respectively. The final interaction and subsequent action of the IGFs is determined by the association of the IGFs with particular binding proteins (see below). Furthermore, the half-life of both IGFs is dramatically extended when complexed with IGF binding proteins [19, 20].

Receptors

There are at least three closely related receptor tyrosine kinases in the insulin receptor family. These are the insulin receptor (IR), the insulin related receptor (IRR), and the IGF1R [18, 34, 35]. Knockout mice experiments have genetically identified a fourth, highly specific, temporally restricted receptor for IGF-2 in development, which is called

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IGFRX due to an overall lack of understanding for the structure, signaling, etcetera, of this receptor [26, 28]. The IR, IRR and IGF1R share several features. They are all $\alpha_2\beta_2$ heterotetrameric transmembrane glycoproteins where the α and β subunits are synthesized from a single mRNA as a large precursor. This precursor is then glycoslyated, proteolytically cleaved, and crosslinked by cysteine bonds to yield the mature $\alpha\beta$ chain. The α-chains are entirely extracellular while the membrane spanning § -chains are responsible for intracellular signal transduction in response to ligand. The mature IGF1R has an apparent molecular weight of 320 kDa. There are two high affinity ligands for the IGF1R, IGF-1 (1nM) and IGF-2 (3nM), that upon binding to the α-chains induce the tyrosine autophosphorylation of the IGF1R §-chains. This receptor autophosphorylation initiates a cascade of signal transduction that results in either growth, survival, or differentiation of the target cell [18, 19]. While the molecules involved in the IGF/IGF1R signal transduction cascade are somewhat cell-type specific, there are some common themes regarding proliferation versus apoptosis/survival. More importantly, IGF signaling has been shown to engender a survival advantage to cells subjected to a variety of potentially lethal insults that include UV and X-irradiation, chemotherapy, oxidative stress, loss survival factors and loss of adhesion [36-48]. The affinity of insulin for the IGF1R is about 100 times lower and *vice versa*. Therefore, the insulin receptor is only activated at supraphysiological concentrations of IGFs and *vice versa*. The ligand for the IRR has not been identified to date.

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The IGF2R, also known as the mannose-6-phosphate receptor, is a 270 kDa glycoprotein that has no homology to the insulin receptor tyrosine kinases. The IGF2R is a bifunctional protein that binds lysozomal enzymes bearing the mannose-6-phosphate recognition site and IGF-2 at different binding sites [49]. To date, no intracellular cell signaling has been found from the IGF2R, suggesting that the IGF2R facilitates the degradation of IGF-2 by transporting IGF-2 to lysozomes, thereby limiting the signal potentiated by the growth factor. Alternatively, the IGF2R may act as a slow release mechanism for excess IGF-2. Indeed, knockout mice for the IGF2R have a 135% increase in body weight compared to control littermates. The importance of the IGF2R is also illustrated by these studies. IGF2R knockouts have numerous developmental abnormalities and usually die perinatally [27, 50].

Binding Proteins

IGFs are transported in plasma and extracellular spaces by high affinity binding proteins. These IGF binding proteins (IGFBPs) range in molecular weight from 25 to 40 kDa [19, 20, 51, 52]. More than 80% of IGFs in circulation are bound to IGFBP-3 in the presence of an 88 kDa non-IGF binding acid labile subunit (ALS) to form a 140 kDa ternary complex. Six high affinity IGF binding proteins (IGFBPs) have been cloned and isolated. These binding proteins are remarkably well conserved. They share at least 50% homology with each other and more than 80% homology between species [53, 54]. Furthermore, with the exception of IGFBP-

6 that preferentially binds IGF-2, the other IGFBPs bind both IGFs with affinities comparable or superior to the receptors . Despite these similarities, many characteristics are different: chromosomal location, RGD recognition site, preference for binding IGF-1 or -2, as well as glycosylation and phosphorylation differences. IGFs bound to IGFBPs represent the reserve pools of IGF. In this context, several major functions are suggested for the IGFBPs [19, 20] : **(1)** to act as transport proteins in plasma and to control the efflux of IGFs from vascular space; **(2)** to prolong the half-lives of the IGFs; **(3)** to provide a means of tissue- and cell- type specific localization; **(4)** to modulate directly the interaction of the IGFs with their receptors and thereby indirectly control biological actions; **(5)** to assist in the regulation of blood glucose levels and **(6)** ligand independent effects of IGFBPs, most notably IGFBP-3, that are mediated through receptors that are distinct from the IGF receptors.

The remainder of this review will discuss recent insights on the role of the IGF axis in prostate neoplasia. The IGF system takes into account most of the complexities observed in prostate pathologies. As discussed briefly above, the IGF axis comprises a complex system of ligands, receptors, binding proteins, and proteases that interact with the extracellular matrix and respond as well as modulate the host endocrine milieu. These characteristics make the IGF axis a valuable and interesting domain for study.

III. THE PROSTATE AND THE IGF AXIS

A. Ligands and Receptors.

IGF-1, IGFBP's and Prostate Cancer Risk

The role of the IGF axis in prostate carcinogenesis is becoming increasingly emphasized. Recent studies show that elevated levels of plasma IGF-1 predict an increased risk of up to six-fold for prostate cancer [55-58]. While a statistical association to prostate cancer is evident there is no direct evidence that elevated serum IGF-1 causes the initiation of prostate cancer. Recent reviews, [57, 58]], interpret the data on elevated IGF levels with the following conclusions. First, IGFs, IGFRs and IGFBPs are expressed in prostatic tissue and IGFs promote growth, while IGFBPs largely inhibit growth. Second, IGFBP-3 has a pro-apoptotic effect in addition to its inhibitory effect on IGF-1 in the prostate. This data has support in the reports by Chan et al.[55] and Hankinson et al. [59] which found a protective effect of elevated IGFBP-3 in patient sera. Third, other binding proteins may be better measures of having a prostate cancer. IGFBP-2 was measured and found to be up to three-fold higher in the serum of prostate cancer patients as compared to normal controls [57, 60-62]. Finally, that elevated serum IGF may contribute to the development of benign prostatic growth, or hyperplasia (BPH), and not to prostate cancer. These conclusions come from studies of growth hormone replacement therapy where no increased risk of prostate cancer was found [57]. That said, in acromegaly, a condition characterized by chronically elevated GH/IGF-1, there is dramatic association with the incidence of BPH but

not prostate cancer [63-65]. Therefore, an elevated level of serum IGF-1 could arise from several sources, including a prostate tumor, and may or may not contribute to the initiation of prostate cancer. It is currently unclear whether or not there is a benefit to be gained using measurements of IGF-1, IGFBP-2 or -3 to predict risk or to aid in the diagnosis of prostate cancer. Finally, the IGF responsiveness of a prostate cancer will be determined by the interaction of the available serum IGF-1 and the expressed milieu of IGF axis components in the prostate, as discussed below.

The IGF axis and Androgens

The maintenance of structure and function in the prostate depends upon the presence of androgens [66] . Furthermore, most metastatic prostate cancer responds favorably to castration [67-69]. However, as prostate cancer progresses it becomes more androgen independent [66, 68-70]. Therefore, another issue concerning prostate cancer is the regulation of the IGF axis by androgens. There are few publications in this area of inquiry. An in vitro study using LNCaP cells showed that very low concentrations of DHT synergized with IGF-1 using thymidine uptake as a readout, while IGF-1 alone had only modest response [71]. In androgen responsive foreskin fibroblasts, IGFBP-3 was not regulated by either IGF-1 or testosterone, but IGFBP-5 was upregulated by IGF and increased synergistically by IGF and testosterone in combination [72]. Interestingly, IGFBP-4 in foreskin fibroblasts was completely degraded in response to IGF-1, while testosterone had no effect on the

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expression or degradation with or without IGF-1. Goosens et al.,[73] showed that the mRNAs for IGFBP-2 and -4 were increased, while IGFBP-3 was repressed in LNCaP cells treated with androgen. PC-3 cells transfected with androgen receptor resulted in a decreased steady-state level of IGFBP-3 protein and mRNA. The transfected PC-3 cells also have decreased cell proliferation rate [74]. The expression of stromal IGF-1 or IGF-2 might be modulated by an androgen-induced peptide, IGFBP-3. In the Dunning rat model, the expression of IGF-1 was induced by androgen in androgen-dependent but not in androgen-independent tumors maintained in castrated hosts [75]. The patterns of expression of the mRNAs were different, depending on the degree of malignancy. In the ALVA prostate cancer cell line, derived from surgical specimens of adenocarcinoma of the prostate, the levels of IGFBP-4 and IGFBP-6 were not modified by androgens [76]. In the androgen-dependent CWR22 prostate cancer xenografts, IGFBP-5 was the only IGFBP regulated by androgens [77]. In the androgen responsive Shinogi tumor model, castration induced rapid increases in IGFBP-5 mRNA and decreases in IGFBP-3 and -4 mRNAs and no effect on IGFBP-2 mRNA levels [78]. The expression level of IGFBP-2, -3, -4 and -5 mRNAs is increased after castration and involution of the rat ventral prostate [79]. These data show that most IGFBPs are androgen repressed in vivo. This study also showed that IGF1R mRNA expression is dependent upon androgen for expression and is lost rapidly after castration. Huynh et al. [80] used the 5∝-reductase inhibitor finasteride to

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demonstrate the dependence of IGF-1 and IGF1R expression on the presence of 5∝-dihydrotestosterone, the active male steroid in prostate tissue, and not simply testosterone. This study also showed an increase in IGFBP-3, further confirming its suppression by androgen in prostatic tissue. Similarly, the use of finasteride demonstrated that IGFBP-5 suppression was due to 5∝-dihydrotestosterone [81]. These findings have now been extended to humans [82]. Men taking finasteride also demonstrated an increase in IGFBP-2, -4 and -5, as well as a decrease in tissue IGF-1. Interestingly, only IGFBP-2 and -4 co-associated with apoptotic markers. All these examples demonstrate a close regulation of the IGF axis by androgens and an involvement of this axis in the evolution towards androgen independence. However, the cell context does appear to determine the ultimate regulation of the IGF axis by androgens and IGF.

IGFs and IGFRs in the Prostate

Pietrzkowski et al. demonstrated the existence of an autocrine loop of proliferation involving IGF-1 and the IGF1R in both androgen-independent human prostate cancer cell lines, DU145 and PC3, as well as in androgen-sensitive cell line, LNCaP [83, 84]. However, these results on the expression of IGFs in prostate epithelial cells are controversial. Subsequent studies could not demonstrate the secretion of IGF-1 by the same prostate cancer cell lines [71, 85, 86] and only very low levels of IGF-1 mRNA in LNCaP [86]. These findings do not completely abrogate the early reports by Pietrzkowski, since an autocrine

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proliferation loop was found in PC3 [87], DU145 [85] and LNCaP cells [86]; however, it is likely due to the expression of IGF-2 as the ligand (See Table 1 for Summary). Furthermore, it is clear that prostate cancer cell lines respond mitogenically to exogenous IGF-1 and bind IGF-1 with high affinity, thereby indicating the presence of the receptor [71, 88]. There have since been several studies [73, 86, 88] (Sikes et al. unpublished observations) to demonstrate the expression of both IGF1R mRNA and protein in prostate cancer cell lines.

Similar studies to determine IGF axis member expression were performed on primary cultures of epithelial and stromal cells derived from normal prostate, BPH, and cancer (Table 1). Epithelial cells and stromal cells express the type I receptor for IGF but not the type II [89, 90]. Normal epithelial cells are sensitive to the mitogenic effect of IGF-1 and IGF-2, but they do not express either ligand [91]. Stromal cells, specifically BPH [40, 91] and cancer [91] stromal cells, produce large amounts of IGF-2 . Another study showed that under different experimental conditions, both epithelial and stromal cells express IGF-2 [92].

There are now several studies that have looked at the expression of IGF axis members in human tissues (Table 2). Figueroa et al. [93], used RNAse protection to show the expression of IGF-2 and IGF1R. Tennant et al. PIN [94-96] used in situ hybridization and immunohistochemistry on tissues to examine the expression of IGFs, IGFRs and binding proteins in prostate cancer samples, BPH and high grade PIN. These

studies demonstrated that IGF-2 is expressed by both stromal and epithelial cells and that the message for this growth factor is increased in prostate cancer. The IGF1R was expressed by both stromal and epithelial cells, but the level was decreased in adenocarcinoma compared to benign epithelium [95]. These results are in contrast with other neoplasia where autocrine proliferation loops often lead to increased expression of the receptor. A partial explanation can be found in the control of IGF-2 expression. The IGF-2 gene demonstrates paternal allele-specific expression, paternal imprinting, and is found in most normal adult and fetal tissues [22]. However, a loss of imprinting for the IGF-2 gene was demonstrated in normal prostate and shown to persist in prostate tumors [92]. A relaxation of this imprinting might explain the overexpression of IGF-2 and the initiation of autocrine stimulation observed in prostate cancer. Finally, IGF-2 has at least two isoforms, a 15 kDa "big" IGF-2 and the more often described 7.5 kDa IGF-2 [97]. "Big" IGF-2 was shown to be more effective at stimulating tyrosine phosphorylation of the IGF1R than either IGF-1 or IGF-2. Furthermore, both IGF-2s were more mitogenic than IGF-1. Interestingly, Li et al. [98] showed that breast, prostate, and bladder cancers expressed the mRNA for the "big" IGF-2 isoform. Similar results were found in a rhadomyosarcoma model system where a 10 kDa form of IGF-2 was found [99]. The question remains to be addressed whether these modifications are a consequence of or are responsible for malignant transformation.

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There is experimental evidence to support the critical involvement of the IGF axis in prostate cancer growth. Pollak et al. [100] showed a decrease in the growth rate of PC-3 xenografts in IGF-1 deficient mice. Antisense RNA to IGF1R has been shown to suppress tumor growth and prevent invasion by rat prostate cancer cells in vivo [101]. IGF-1 analogs that block IGF1R activation prevent prostate cancer cell growth [84]. Neutralizing antibodies to the IGF1R also prevent prostate cancer cell growth [86]. Therefore, comprehension of the tight regulations and interactions of IGF axis will probably give new insights to the understanding of prostate cancer.

B. Binding proteins and proteases

IGFBP-1

Introduction

The human gene for IGFBP-1 is located on chromosome 7 [102] and encodes a 25 kDa protein that contains an RGD motif. The RGD motif is recognized as a determinant for integrin binding suggesting that ligand-independent effects of IGFBP-1 could be mediated through integrin heterodimers [103]. In Chinese hamster ovary cells IGFBP-1 stimulates migration that can be abolished by mutating the IGFBP-1 RGD motif [104]. IGFBP-1 could also be co-immunoprecipitated with antibodies to the $\alpha5$ integrin subunit that cemented the involvement of IGFBP-1 in integrin mediated migration. IGF-1 had no effect on this activity. IGFBP-1 is expressed in the placenta [105] where it may limit trophoblast invasion. It is expressed in skeletal myoblasts, where it modulates differentiation into myotubes

[106]. IGFBP-1 is also made by the liver, where its expression is suppressed by insulin and increased by glucagon [107]. IGFBP-1 is therefore tightly regulated by nutritional status [108] and is thought to regulate tightly the pool of free IGF-1 and its half-life in circulation [109]. Furthermore, IGFBP-1 is found in several phosphorylation states that determine its affinity for the IGFs [110, 111]. Phosphorylated IGFBP-1 has a six-fold higher binding affinity than the unmodified binding protein. Furthermore, it is the phosphorylated IGFBP-1 that has been implicated in growth inhibition. Interestingly, at this time the change in affinity for IGF has only been observed for the human IGFBP-1 and not for the rat.

IGFBP-1 in the prostate

IGFBP-1 has been used to demonstrate the growth inhibitory effects of IGFBPs on IGFs in prostate cancer cell growth and the existence of an autocrine proliferation loop [85]. However, there has been no description of IGFBP-1 expression in prostate tissues or cell lines to date [40, 73, 87, 89, 91, 93, 112]. Koutsilieris et al. [113] isolated a serine protease activity from the prostate tumor cell line PA-III that cleaves IGFBP-1 and -2. This activity increased the growth of osteoblastic and PA-III cells and could be blocked by antibodies to IGF-1. Therefore, while not directly made by the prostate or prostate cancer cells, IGFBP-1 may have a role in the regulation of prostate cancer behavior in most frequent site of prostate cancer metastasis, the bone.

IGFBP-2

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Introduction

The gene for IGFBP-2 is located in chromosome 2 [102] and encodes a 31 kDa protein [19]. IGFBP-2, like IGFBP-1, contains an RGD motif, but no binding to integrin receptors has been documented to date. There are also no reports of IGFBP-2 being either glycosylated or phosphorylated. In the 13.5 day post-coital mouse, IGFBP-2 expression is localized to the lungs, liver, kidney, choroid plexus and floor plate [114]. In most cases the mRNA and protein co-localize; however, in the tubule of the kidney the mRNA for IGFBP-2 is in the epithelium and the protein is localized in the stroma. IGFBP-2 is increased in the follicular fluid from patients with polycystic ovary disease, suggesting a stimulatory role in this tissue [115]. IGFBP-2 is overexpressed in glioblastoma multiforme [116], leukemic T-cells [117], adrenocortical tumors [118], neuroectodermal tumors [119], and cell lines from breast cancer [120-122], Wilm's tumor [122], colon cancer [122], Burkitt's lymphoma [122], and lung cancer [122, 123].

IGFBP-2 Modulation of IGF Action and Expression in Prostate

Transgenic animals overexpressing IGFBP-2 have markedly reduced body weight, suggesting a largely antagonistic role for IGFBP-2 in mediating the effects of the IGFs. Likewise, retinoic acid suppression of IGFBP-2 corresponds to increased mitogenicity in human lung alveolar cells [124]. This is clearly context dependent, as IGFBP-2 enhanced the mitogenic effects of IGF-1 in the MCF-7 breast cancer cell line [125]. Similarly, in

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estrogen-receptor negative breast cancer cell lines, where IGF-1 stimulated cell growth, IGFBP-2 was upregulated [121]. Also, many colon cancer cell lines express IGFBP-2 at some level [122]. Furthermore, overexpression of IGFBP-2 in an epidermoid carcinoma resulted in elevated levels of IGF, increased tumor take and growth [126]. More recently, increased IGFBP-2 levels were associated with the development of an androgen-independent phenotype in the Shionogi tumor model that further suggest a stimulatory role in cancer progression [78]. BPH is a benign proliferative disease of the prostate characterized by the overexpression of IGF-2 by stromal cells and a decreased expression of IGFBP-2 with a concomitant increase in IGFBP-5, which is not expressed in normal prostate (Table 1) [91]. These changes were presumed to regulate the bioavailability of IGFs in favor of growth stimulation, since IGFBP-2 had the tendency to inhibit IGF action in stromal cells, whereas IGFBP-5 stimulated IGF effects. Similarly, in neuroblastoma cells, IGFBP-2 appears to be inhibitory as retinoic acid (RA) treatment initiates rapid degradation of IGFBP-2 and an increased synthesis of IGF-2 [127]. As RA promotes cell growth both the proteolysis of IGFBP-2 and the increased IGF-2 synthesis would serve to increase the free pool of IGF.

IGFBP-2 was shown to have altered levels of expression in prostate pathologies (Table 2). In contrast to BPH, the level of IGFBP-2 in patient serums is increased in prostate cancer [61]. Tennant and colleagues [96] analyzed IGFBP expression at both the protein and mRNA levels in prostate tissues. They showed that both IGFBP-2 protein and mRNA were

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increased in PIN and increased further in adenocarcinomas as compared to benign tissue. This may reflect different mechanisms of action for IGFBP-2 in stromal versus epithelial cells in the prostate. Recently, cDNA expression arrays coupled with tissue microarray analysis have determined that IGFBP-2 is overexpressed in 100% of hormone-refractory prostate cancers, 36% of primary prostate cancers and none of the benign samples tested [128]. IGFBP-2 has the potential to associate with cell surface ligand (or recognition site) through a RGD sequence. Binding of IGFBP-2 to an integrin receptor may facilitate transport of IGF-1 to the type I IGF receptor or may stimulate cell motility as shown for IGFBP-1 [104]. IGFBP-2 was also shown to bind to heparan and ECM when complexed with IGF-1 or IGF-2 [129]. This is very interesting when one considers that many growth factors known to stimulate prostate epithelial cells are heparin binding, including some unknown factors produced by an osteosarcoma cell line [130].

Proteolysis of IGFBP-2

The degradation of IGFBP-2 has been associated with a decreased affinity for IGFs, thereby providing a mechanism for overcoming the growth inhibitory role of this binding protein [20]. In neuroblastoma the proliferative effects of bFGF have been attributed to the degradation of IGFBP-2 by a metalloproteinase [131]. Several groups have shown that Cathepsin D can mediate IGFBP proteolysis in prostate cancer cell lines [112, 132]. While this is largely considered a lysomal protease active at acidic pH, it has been found in the conditioned media from several cancer cell

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lines. Furthermore, the surface of cancer cells appears to be highly acidic in nature and corresponds with metastatic potential in rat prostate cancer cells [133]. In the lung, matrix metalloproteinase 1 (MMP-1) is responsible for the degradation of IGFBP-2 [134]. Overexpression of this MMP probably plays a role in asthma. Koutsilieris et al. [113] showed that rat prostate cancer cells (PA-III) secrete urokinase plasminogen activator that degrades IGFBP-2 from osteoblasts. Other metastatic prostate cancer cell lines also secrete urokinase which has been associated with an increased metastatic potential [135-139]. Therefore, expression of IGFBP proteases like urokinase, MMPs, and kallikreins (PSA, hK2) may facilitate prostate cancer metastasis to bone.

Regulation of IGFBP-2 Expression

IGFBP-2 is positively regulated by IGF-1 and negatively regulated by retinoic acid (RA) in estrogen receptor negative breast cancer cell lines [121]. RA also decreases expression of IGFBP-2 in neuroblastoma, although the regulation is by proteolysis and decreased mRNA stability and not through protein synthesis, secretion, or transcriptional regulation [127, 140]. Castration induces the expression of IGFBP-2 in the rat [79], but this cannot be due to the action of the biologically active androgen 5α-DHT since the administration of finasteride cannot reproduce this effect [80]. In humans, however, finasteride administration does result in an upregulation of IGFBP-2 levels [82]. Goosens et al. [73] found an upregulation of IGFBP-2 mRNA in response to low dose androgen treatment in the LNCaP human prostate cancer cell line. It is not clear

whether this is associated with an increase in IGFBP-2 protein expression or not. Hypoxia has been shown to upregulate the expression of IGFBP-2 and IGF1R in the retina [141]. Tumors ultimately outgrow their vascular support which leads to hypoxic conditions [142]. Many genes upregulated by hypoxia are targeted towards neovascularization, like vascular endothelial growth factor (VEGF) [142-146]. Interestingly, VEGF is also regulated by IGF-1 in colon cancer [147]. In the LNCaP progression model of human prostate cancer [148, 149] there is increasing VEGF production along with increasing IGF-2 as the cells become more androgen independent [150] (Sikes et al. Unpublished observations, Simons et al., Personal communication).

IGFBP-3

Introduction

The gene for human IGFBP-3 is located on chromosome 7 in close proximity to the gene for IGFBP-1 [102]. IGFBP-3 is a 45-50 kDa protein containing three N-linked glycosylation sites and a nuclear localization signal (NLS) sequence. IGFBP-3 is the major circulating IGFBP and the main IGF carrier in serum. Free IGFBP-3 has a high affinity for IGF-I and II and most of the serum IGF-I circulates as a 150 kDa complex with IGFBP-3 and an acid labile subunit [19]. This high affinity binding allows IGFBP-3 to sequester IGF-I, preventing IGF-I binding to its cognate receptor, thus blocking IGF-I action and causing IGFBP-3 to inhibit cell growth. Early studies showed that transfection of the IGFBP-3 gene into Balb/c fibroblasts inhibited cell growth [151] and that IGFBP-

3 blocked IGF-I stimulated DNA synthesis in human skin fibroblasts [152]. Senescent fibroblasts were shown to overexpress IGFBP-3 [153, 154]. Cohen and colleagues demonstrated that IGFBP-3 induced apoptosis and mediated TGF-β1-induced apoptosis in prostate cancer PC-3 cells [155, 156]. Moreover, IGFBP-3 was shown to decrease proliferation and to modulate retinoic acid- and TGF-β2-induced growth inhibition in breast cancer cells [157, 158].

IGFBP-3 in Growth and apoptosis

IGFBP-3 was detected in conditioned medium from human prostate epithelial cells [159] and from prostate stromal cultures [90, 91] (Table 1). The androgen-independent prostate cancer cell line PC-3 was also shown to secrete IGFBP-3 in addition to IGFBP-2, -4, and -6 [87]. In PC-3 cells, proteolytic fragments of IGFBP-3 were prevalent. The authors suggested that the biological activity of IGF-I and -2, which are normally bound to IGFBP-3, may be directly related to the proteolysis of the binding protein. That is to say, increased IGFBP-3 proteolysis prevents it from binding to serum IGFs, thus allowing IGFs to activate signaling pathways in the cell to induce growth. In contrast, PC-3 cells stably transfected with a constitutively active androgen receptor did not secrete IGFBP-3. However, when treated with IGFBP-3/IGF-I in combination, the cells increased their proliferation rate, suggesting that higher concentrations of IGFBP-3 may result in an enhancement of the IGF-I response [74].

IGFBP-3 may be a direct inducer of apoptosis in both breast and prostate

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cancer, independent of IGF-I effects. Incubation of breast cancer cells with a non-glycosylated IGFBP-3 sensitized the cells to ceramide-induced apoptosis in an IGF-independent manner [160]. Similarly, IGFBP-3 caused apoptosis in prostate cancer PC-3 cells independent of the IGF-IGF receptor and p53 pathways [156]. IGFBP-3 was implicated as a serum component likely responsible for high-serum induced apoptosis in PC-3 cells [155].

Proteolysis of IGFBP-3

Proteolytic fragments have lower affinity for IGFs, thus allowing increased levels of free IGFs to bind to IGF-I receptors. Prostate-specific antigen was the first described IGFBP-3 protease [161]. The kallikrein-like serine proteases, the cathepsins [112] and the matrix metalloproteinases [162] all have been shown to cleave IGFBP-3. It is interesting to note that all three of these protease families are expressed in prostate and/or prostate cancer. Some of these proteases were not found in the carcinomatous elements but rather were located in stromal cells producing NGF [163] or other stromal components, as described in other organs [164]. Recently a direct role of MMP-9 was assessed in the autocrine proliferation of DU145 cells. The IGF-1 autocrine proliferation loop is regulated by IGFBP-3 whose activity may in turn be regulated by IGFBP-3 proteases, in particular MMP-9 [162]. Decreasing the IGF binding activity of IGFBP-3 by proteolytic cleavage causes a dysregulation of the IGF signaling pathways in prostate cancer cells, likely leading to induction of cell growth. These

examples illustrate the existence of a balance between autocrine secretion of IGF ligands, IGFBPs, and proteases that are disrupted in carcinogenesis.

Regulation of IGFBP-3 expression

IGFBP-3 expression was increased in PC-3 cells by treatment with TGF-β and retinoic acid, which are known inhibitors of prostate cell proliferation [165]. Androgen was reported to be an important modulator of IGFBP-3 expression as well. In the rat ventral prostate, castration caused increased expression of IGFBP-2, -3, -4, and —5 with maximum levels reachedat 72 hours after castration [79]. The 5α-reductase inhibitor finasteride caused a decrease in rat ventral prostate weight. A concomitant increase in IGFBP-3 expression and decrease in IGF-I and IGF-I receptor expression was also observed suggesting that finasteride may suppress prostate growth by inhibition of IGF signaling pathways [80]. In LNCaP prostate cancer cells, the synthetic androgen methyltrienolone (R1881) caused the downregulation of IGFBP-3 mRNA while retinoids (all-*trans-* and 9-*cis*-retinoic acid) caused upregulation. These data suggest that the regulation of IGFBP-3 by nuclear steroid hormone receptors may be complex and receptor specific [73]. In contrast, dihydrotestosterone and 1,25-dihydroxyvitamin D_3 in combination increased IGFBP-3 production by cultured LNCaP cells [166]. Increased IGFBP-3 was shown to stimulate DNA synthesis and cell proliferation in LNCaP cells. In the CWR22 human prostate cancer xenograft model, IGFBP-3 mRNA did not change significantly after castration or with testosterone treatment and was not

increased in the recurrent tumor [77]. Therefore, studies in cell lines, rat ventral prostate and human prostate cancer xenografts gave somewhat disparate results. Nonetheless, the data suggest that IGFBP-3 function is important for prostate cancer growth (in a positive or negative manner).

Clinical significance of IGFBP-3

Circulating IGF-I levels were significantly related to prostate cancer risk while serum IGFBP-3 showed a small inverse association with risk that was stronger in older men [55]. In a second study from Sweden, IGFBP-3 was not associated with prostate cancer risk [56]. Serum IGFBP-3 levels were shown to differ significantly in Caucasian vs. African-American prostate cancer patients with a mean IGFBP-3 plasma level that was 427 ng/ml lower in African-American men [167]. This lowered circulating IGFBP-3 could lead to greater bioavailability of IGF-I, possibly resulting in increased proliferation of prostate cancer cells.

IGFBP-4

Introduction

The gene for IGFBP-4 is located on chromosome 17 [102] and encodes a protein of 24 kDa or 28 kDa when glycosylated [20]. IGFBP-4 has been found in bovine and human fibroblasts [72, 168, 169], endothelial cells [170], spinal cord [171], osteoblasts and bone mesenchyme but not condensing mesenchyme [172-175], muscle [176] and prostate [79, 177, 178] among others. Further, IGFBP-4 has been found in lung cancer [179],

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neuroblastoma cell lines [127, 140], breast cancer cells [121, 180], osteosarcoma cell lines [175, 181] and prostate [73, 82, 178]. Therefore, IGFBP-4 is expressed in a number of tissues and cell lines of diverse origin having specific patterns of expression throughout development. Of particular interest to prostate cancer is the expression and regulation of IGFBP-4 in bone-derived cell lines and the impact on prostate cell proliferation.

IGFBP-4 Modulation of IGF Action and Expression in Prostate

IGFBP-4 appears to inhibit most of the actions of IGF in vitro [19, 20]. The overexpression of IGFBP-4 in myoblasts prevents IGF-1 mediated myotube differentiation [176]. The removal of IGFBP-4 from the conditioned media of a colon cancer cell line by the addition of antibodies against IGFBP-4 stimulated mitogenesis [182]. Similarly, the addition of exogenous IGFBP-4 directly prevented IGF-1 or -2 binding to the IGF1R in a dose-dependent manner using human osteogenic sarcoma cell lines [183]. Exogenous IGFBP-4 decreased the mitogenic response of vascular smooth muscle cells to IGF-1 [184]. Noll et al. [179] showed that IGF-1 increased growth in non-small cell lung carcinoma cell lines. Furthermore, the IGF effects were directly associated with the degradation of IGFBP-4. Indirect evidence for the inhibitory role of IGFBP-4 comes from studies on the P69 lineage from Plymate et al. [88]. This study found IGFBP-4 expressed in primary cultures of prostate epithelial cells but progressively reduced expression in immortalized and aggressively tumorigenic cell lines derived from the primary cultures.

A direct examination of the effects of IGFBP-4 expression in prostate model systems has not been described to date. In summary, every study to date demonstrates an inhibitory effect of IGFBP-4 on IGF-mediated events.

In prostate cancer cell lines (Table 1) IGFBP-4 protein was found in Du145 [86, 87] and PC-3 [86] conditioned media. In the study by Kimura st al. [86] LNCaP cells had no IGFBP-4 as ascertained by western ligand blot and an abnormally large band in western blot. Goosens et al. [73] showed very weak expression of IGFBP-4 mRNA in LNCaP cells. As discussed above, P69 and the metastatic derivative, M12, continue to express IGFBP-4 in vitro, albeit at lower levels than in primary cultures of prostate epithelial cells [88]. Similarly, Cohen et al. [89] demonstrated the expression of a 24 kDa IGFBP in primary cultures of prostate epithelial cells that is probably IGFBP-4. Primary cultures of prostatic stromal cells also express IGFBP-4 [91]. However, the levels of IGFBP-4 secreted were variable with no detectable trend in expression between normal, BPH and cancerous stromal cells. Overall, these data would indicate a decrease in the expression of IGFBP-4 in accordance with the aggressiveness of the prostatic neoplasm, but a direct inhibitory role has not been demonstrated.

The expression of IGFBP-4 in prostate tissues (Table 2) has not been as extensive as for other IGFBPs. The expression of IGFBP-4 mRNA has been documented in both BPH and prostate cancer [93, 94, 177, 178]. These studies show that IGFBP-4 mRNA is located in

both the epithelial and stromal compartments, but the epithelial component expresses much more than the stromal compartment. The expression levels increased slightly from normal and BPH tissue when compared to cancer [94], but this association does not seem to hold with increasing Gleason grade [93]. Therefore, the modest increases in IGFBP-4 protein expression and negative growth effects are probably exceeded by the increases observed for IGF-2, IGFBP-2, and -5 positive growth signals [93-95, 98, 185].

Proteolysis of IGFBP-4

The degradation of IGFBP-4 appears to be a common mechanism to decrease its levels and prevent the inhibitory effects of this binding protein on IGF action. In osteosarcoma cells estradiol increases IGFBP-4 proteolysis [186]. Human fibroblasts, osteosarcoma, and lung cancer all secrete IGFBP-4 proteases in response to IGF [179, 187]. That this activity was EDTA sensitive in lung cancer cell lines indicates the involvement of a matrix metalloproteinase (MMP). Similar indications of MMP involvement in IGFBP-4 degradation were found in pregnancy serum [20, 188-190]. Prostate specific antigen was shown to cleave IGFBP-4 and -5 in seminal plasma [191]. Interestingly, another kallikrein family member related to PSA, gamma-NGF, has demonstrated potent proteolytic activity towards IGFBP-4 [20, 163]. Prostate epithelial cells, but not stromal cells, secrete acid activated proteases [132], probably cathepsins [20, 112], that rapidly degrade IGFBP-4. The degradation of IGFBP-4 decreases the inhibitory action of this IGFBP by liberating

bound IGF and preventing further sequestration of IGFs.

Regulation of IGFBP-4 expression

As discussed above, IGFBP-4 inhibits osteosarcoma growth [192]. IGFBP-4 is downregulated by bone morphogenetic protein-7 (BMP-7, OP-1) in osteoblasts and osteosarcoma [193]. BMP-7 and IGF-1 stimulate osteoblasts and osteosarcoma [192, 193] and both are overexpressed in osteosarcoma samples [194]. Furthermore, the mouse prostate expresses BMP-7 in an androgen-dependent manner and LNCaP cells express the receptor for BMP-7 [195, 196]. Given the predilection of prostate cancer to metastasize to the bone and its osteomimetic properties [136], this BMP-7 network would provide an excellent regulatory pathway to suppress IGFBP-4 in prostate cancer cells. IGF-1 has been shown to decrease IGFBP-4 expression in human fibroblasts [168, 169], colon cancer [197], lung cancer [179], and breast cancer [120]. This is consistent with the increased expression of IGF-2 and negligible change in IGFBP-4 levels in prostate cancer as discussed above. IGFBP-4 expression is associated with cell culture density in vascular smooth muscle cell cultures [184], which may provide the explanation for the decreased proliferative response of these cells to IGF-1 at high density. In contrast to the BMP data, agents that have a differentiative effect in colon cancer, namely estradiol, retinoic acid, and vitamin D, all increase IGFBP-4 levels [197]. This is cell type specific since human osteogenic sarcoma cells, SaOS-2, actually turnover or decrease IGFBP-4 in response to estradiol [186]. Goosens et al.

[73] showed that very weak expression of IGFBP-4 mRNA in LNCaP cells was mildly stimulated by androgen. Additional studies on prostate cell lines are not available; however, prostatic involution in rats [79, 177], and humans [82] following androgen ablation results in increased levels of IGFBP-4 mRNA and protein. This would argue that IGFBP-4 is repressed by androgen. Prostate cells have a biphasic growth response to androgens where low and high doses are differentiative, while moderate doses are proliferative [198]. This may explain some of the paradoxical effects of androgens on IGFBP expression.

IGFBP-5

Introduction

The gene for human IGFBP-5 is located on chromosome 2 [102]. IGFBP-5 is a 33-35 kDa protein that migrates as a doublet on sodium dodecyl sulfate polyacrylamide gels, likely due to glycosylation. IGFBP-5 is expressed in a number of tissues and cell lines, including rat osteoblast cells [199], osteosarcoma cells [200], ovarian granulosa cells [201], articular chondrocytes [202], human fibroblasts [203], porcine smooth muscle cells [204], mouse brain [205], rat thyroid [206], and rat mammary gland [207]. IGFBP-5 has also been implicated in prostate cancer progression, but the specific functional role of IGFBP-5 has not been clearly defined. IGFBP-5 is expressed by the rat ventral prostate [79, 81], LNCaP and PC3 cultured cells [112], the CWR22 human prostate cancer xenograft, the Shinogi mouse mammary tumor model [77, 78], as well as

benign and malignant human prostate tissue [77, 82, 93, 94](see Tables).

Inhibition of IGF-I actions by IGFBP-5

Proteolysis of IGFBP-5 reduces its affinity for IGF-I, thereby modulating the interaction of the binding protein and the ligand [208]. Early work demonstrated that a serine protease secreted by human fibroblasts effectively cleaved IGFBP-5 [208]. Tindall and colleagues showed that endogenous cathepsin D caused hydrolysis of IGFBP-2, -3, -4, and -5 produced by cultured prostate cancer cells [112]. Thrombin, a serine protease present in extracellular matrix (ECM) of human tissues, degraded only IGFBP-5 [209]. IGFBP-5 binds to the ECM; however, not all of the ECM proteins involved have been identified. Plasminogen activator inhibitor-1 (PAI-1) is a constituent of the ECM, and it was shown to bind IGFBP-5 and partially protect it from proteolysis [210]. Preventing IGFBP-5 binding to the ECM inhibited DNA synthesis in response to IGF-I in smooth muscle cells [211]. An excess of intact IGFBP-5 also inhibited IGF-I action in another study of smooth muscle cells and human osteosarcoma cells [200, 212].

Stimulation of IGF-I actions by IGFBP-5

IGFBP-5 potentiates IGF-I action in smooth muscle cells if proteolytically degraded, although the fragments have low affinity for IGF-I [203]. A fragment of IGFBP-5 purified from cultured U2 osteosarcoma cells augmented the mitogenic effect of IGF-I on cultured mouse osteoblasts [213]. Gleave and colleagues demonstrated that overexpression of IGFBP-5 in LNCaP cells

increased growth rates in the presence and absence of IGF-I [214]. Likewise, treatment of Shinogi mouse mammary tumor cells with IGFBP-5 antisense oligonucleotides inhibited growth of the cells, and this could be reversed by IGF-I treatment [215]. Jones et al. [203] described the IGF potentiating effects of ECM-associated IGFBP-5 in human fibroblasts. These findings suggest that the antiapoptotic and growth-promoting effects of IGF-I could be modulated by IGFBP-5 in prostate cancer progression and that IGF-I activity could be enhanced through upregulation of IGFBP-5, probably via paracrine interactions with adjacent stroma.

Regulation of IGFBP-5 and Expression in Prostate

Previous studies on the regulation of IGFBP-5 have been confined largely to cell lines. Both IGF-I and IGF-II stimulate IGFBP-5 expression [121, 169, 216]. In some cell types, IGF-I increases secretion of IGFBP-5 protein without detectable changes in mRNA [121, 169, 199, 202]. In other cell types [199, 216] both the mRNA levels and secretion of IGFBP-5 are increased. Testosterone was shown to enhance the stimulatory effect of IGF-I on IGFBP-5 mRNA and protein in cultured human genital skin fibroblasts [72]. IGF-I was shown recently to regulate IGFBP-5 gene expression through the phosphatidylinositol 3- kinase, protein kinase B/Akt, p70 S6 kinase signaling pathway in vascular smooth muscle cells [217]. IGFBP-5 expression was induced in cultured rat osteoblasts by 1α, 25-dihydroxyvitamin D3 and parathyroid hormone [218]. IGFBP-5 production was

increased fifty-fold in milk after 2 days of rat mammary gland involution induced by removing the suckling pups. This increase was inhibited by 90% if prolactin was administered to the rats. IGFBP-5 synthesis was unaffected by growth hormone, progesterone, or corticosterone in rat mammary glands [207]. Sunic et al. demonstrated that IGF-I and interleukin-1α synergistically increased IGFBP-5 levels in ovine articular chondrocytes, with IGF-I inhibiting proteolysis and interleukin-1α stimulating expression [202].

The regulation of IGFBP-5 expression and function in prostate cancer has been explored using cell lines, xenografts and human tissue. Transforming growth factor β-2 increased IGFBP-5 expression in the PC-3 cell line, whereas retinoic acid treatment resulted in downregulation of IGFBP-5 mRNA [165]. IGFBP-5 transcripts were upregulated rapidly in the rat ventral prostate with peak expression at 72 h after castration [79]. Protein levels for IGFBP-5 in the rat ventral prostate reached maximum expression levels at 9 days after castration, as determined by immunostaining [81]. The association of IGFBP-5 expression with apoptosis caused by androgen withdrawal has been postulated, but a direct functional link has not been described. Increased expression of IGFBP-5 mRNA was associated with apoptotic regression of Shinogi mouse mammary tumors following castration [78]. In contrast, while IGFBP-2 and -4 were increased significantly in involuting prostates of men taking the 5α-reductase inhibitor finasteride, IGFBP-5 protein was not increased and did not co-localize with apoptotic markers [82]. IGFBP-5 mRNA, protein expression and ligand binding

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activity were decreased following castration in the CWR22 human prostate cancer xenograft model. These were elevated, however, in recurrent CWR22 growing in castrated mice [77], suggesting an association of IGFBP-5 function with progression of prostate cancer. Gleave and colleagues confirmed these findings in both LNCaP tumors and in the Shinogi model where the overexpression of IGFBP-5 resulted in a faster progression to androgen independence and the growth of tumor cells could be inhibited by treatment with IGFBP-5 antisense oligonucleotides [215]. Therefore, while IGFBP-5 expression appears to be responsive to androgen status in prostate cancer cells and tumors, the direct functional role of IGFBP-5 is not clearly understood.

A possible clue to IGFBP-5 function in benign and malignant human prostate relates directly to the subcellular localization of the mRNA and the protein (Table 2). Stromal-epithelial cell interactions are likely important in normal prostatic development and in the progression to prostate cancer [219]. Plymate and colleagues demonstrated immunostaining for IGFBP-5 protein in epithelium and stroma of benign tissue with increased expression in malignant compared to benign epithelium. The mRNA was localized to the stroma surrounding the acinar glands in benign tissue, prostatic intraepithelial neoplasia, and prostate cancer [94]. Likewise, IGFBP-5 mRNA was localized to the stromal cells of benign prostate and prostate cancer with IGFBP-5 protein expression found exclusively in epithelial cells. Previous studies support these data [91, 220]. There was a 6 to 8-fold increase in protein

expression in androgen-dependent and recurrent prostate cancer compared to benign tissue [77]. These findings suggest that IGFBP-5 mRNA is transcribed in the stromal compartment in prostate tissue, but the mRNA is translated into protein that is sequestered by the epithelial cells. Expression of IGFBP-5 by the epithelial component may then enable these cells to bind IGF-I, resulting in mitogenic effects causing cell proliferation. Increased IGFBP-5 protein expression in prostate cancer epithelium would thereby enhance cell proliferation and, in the recurrent state, enable cells to grow in the absence of testicular androgen.

IGFBP-6.

Introduction

The human gene for IGFBP-6 is located on chromosome 12 [102] and encodes a protein of 34 kDa [20, 53]. IGFBP-6 is an O-glycosylated protein which, unlike the IGFBP-1 to -5, binds IGF-2 preferentially [221]. In normal tissue or non-transformed cells, IGFBP-6 has been found in osteoblasts [193, 222], ovarian tissue [19], fibroblasts [223] and primary cultures of prostate epithelial cells [88]. In neoplastic tissues or cell lines IGFBP-6 has been found in neuroblastoma [127, 140], osteosarcoma [37], breast cancer [121], and prostate cancer cell lines and tissues [73, 76, 86-88, 93, 94, 177].

IGFBP-6 Modulation of IGF Action and Expression in Prostate

Due to its extraordinarily high affinity for IGF-2 [221], IGFBP-6 does not affect IGF-1 mediated events. In contrast, excess IGFBP-6 has been shown to decrease

IGF-2 mediated DNA synthesis in osteoblasts [221, 223]. IGFBP-6 is induced by IGF-2, which then sequesters IGF-2, thereby dampening the IGF-2 signaling in a negative feedback loop [127, 223]. There is one report of IGFBP-6 stimulating the growth and survival of osteosarcoma cells without binding to the cells or being degraded [37], while similar effects were not observed using normal rat osteoblasts as targets. IGFBP-6 has been found in PC-3, DU145, ALVA-31 and P69 prostate cell lines [76, 86, 88] but not in LNCaP cells [73, 86] (Table 1). Interestingly, as the P69 cell lines become more aggressive they lose expression of IGFBP-6 [88]. Similarly, IGFBP-6 is expressed in PC-3 and DU145 prostate cancer cell lines that have an autocrine proliferation loop involving IGF-2 [86]. Therefore, IGFBP-6 expression may be an attempt by these cells to slow their growth.

In human prostate tissues (Table 2) IGFBP-6 mRNA was found in normal, BPH and prostate cancer [73, 93, 94]. IGFBP-6 has also been found in the rat ventral prostate using cDNA microarrays [177]. Several studies have analyzed the expression of IGFBP-6 in prostate adenocarcinoma versus BPH and PIN [73, 93, 94]. Overall, there was no difference in IGFBP-6 expression among benign and adenocarcinoma tissues, regardless of Gleason grade [93], although both the mRNA and protein were expressed predominantly in the epithelium [94]. It is interesting to speculate that IGFBP-6 levels correspond to the observed levels for IGF-2 expressed in these prostate neoplasms [95, 98]. Additional efforts will also have to determine whether IGFBP-6 has a similar affinity for the large forms of

IGF-2 that were shown to be more potent mitogens in cancer [97, 98].

Proteolysis of IGFBP-6

Gamma-NGF, a PSA-related kallikrein, demonstrated potent proteolytic activity towards IGFBP-6 [20, 163]. While, gamma-NGF may synerigize with the IGF system by binding to its own receptor and degrading IGFBP-6 to release IGF-2, it is not expressed in the prostate [15]. Therefore, degradation of IGFBP-6 appears to be a minor mechanism regulating bioavailable IGF-2.

Regulation of IGFBP-6 Expression

IGF-2, as discussed above, upregulates the expression of IGFBP-6 in [127, 223]. The upregulation of IGF-2 appears to be the mechanism behind the upregulation of IGFBP-6 by retinoic acid in neuroblastoma cell lines [127, 140]. While retinoic acid likewise increases IGFBP-6 expression in estrogen receptor negative breast cancer cells, it remains unclear whether this is also mediated by increased IGF-2 expression [121]. Members of the TGF-§ family of proteins downregulate IGFBP-6 expression in osteoblasts [193, 222]. At least in one study this could be directly attributed to transcriptional suppression [222]. However, this same study showed that TGF-§1 also downregulated IGF-2, which, as discussed, is a potent positive regulator of IGF-2. These effects are consistent with the effects of TGF-β1 to inhibit osteoblast growth and promote differentiation. This seems to be the case for BMP-7, also known as osteogenic protein 1 [193]. BMP-7 also dramatically suppresses IGFBP-6 levels, thereby

promoting osteoblast differentiation. The mouse prostate expresses BMP-7 [195] and prostate cell lines express TGF-β receptors [16, 196]. The suppression of IGFBP-6 through this mechanism would allow for prostate cancer cells to respond to the bioavailable IGF-2. In contrast, other differentiation promoting compounds (e.g. Vitamin D) have also been shown to increase IGFBP-6 levels in ALVA prostate cancer cells [76]. This may provide a mechanistic basis for the function of this class of compounds in the prostate. Other steroid hormones may also play a role in the regulation of IGFBP-6 in the prostate. Bruyninx et al. [177] recently showed that IGFBP-6 mRNA is upregulated during castration of the rat ventral prostate. The implication is that androgenic steroids play some role in the suppression of IGFBP-6.

mac25/IGFBP-rP1.
Introduction

The gene for mac25 is located on chromosome 4q12-13 [224] and encodes a protein of 27-31 kDa non-reduced [225] or about 37 kDa when reduced [226]. This protein was cloned by several groups simultaneously and was found to be a unique IGFBP-related protein (IGFBP-rP1) having significant homology to follistatin [224, 227]. Mac25 was cloned from normal leptomeningial and mammary epithelial cells; its expression was found to be decreased in the corresponding tumor cells [228, 229]. The protein is able to bind IGF with a weak affinity [230]. During the same period, two other proteins were purified that have been determined to be the same as the protein encoded by mac25. TAF (tumor adhesion factor) was

isolated from diploid fibroblasts [231], while PSF (prostacyclin-stimulating factor) was purified from human bladder carcinoma cells [232]. Finally T1A12 was identified by subtractive cDNA cloning using RNAs from a normal breast epithelial cell line Hs578Bst and the breast cancer cell line Hs578T [233]. These were all ultimately found to be the same gene.

Regulation of mac25/IGFBP-rP1 expression

Originally, the expression of mac25 was thought to be ubiquitous due to the presence of mac25 RNA in all tissue samples examined [230]. However, an examination of the protein expression in human tissues undertaken by Degeorges et al. [226] clearly demonstrated that the ubiquitous expression in most tissues was due to strong expression in peripheral nerve cells. In addition, all smooth muscle cells were positive, as were most endothelial cells. Finally, there were specific patterns of immunoreactivity in ciliated structures, kidney tubules, and central nervous system. Immunoreactivity was demonstrated in normal mammary epithelium but not in breast adenocarcinomas, leading to the hypothesis that IGFBP-rP1 might have a tumor suppressor-like function in breast [224]. Chromosomal loss of heterozygosity (LOH) of chromosome 4 in breast cancer accompanies the loss of mac25 expression and lends some support to this theory. In contrast to these reports for breast, studies on mac25 expression in myogenesis show that mac25 levels are highest in proliferating myoblasts and lowest in differentiated myotubules [234]. Indeed, constitutive elevation of mac25 in

myoblasts prevents differentiation into myotubules and alters myoblast response to IGF-1 [106]. In support of this, TGF-β, which prevents myoblast differentiation, elevates mac25 levels, while IGF-1, which stimulates myotubule formation, decreases mac25 expression [234]. Finally, the expression of mac25 by breast cancer cell lines is mixed with some aggressive breast cancer cell lines expressing high levels of mac25 mRNA [228, 230]. The normal prostate, in direct contrast to results observed in breast tissue, did not display any positive immunoreactivity in glandular epithelium and only stochastic expression in the basal cell layer. We then stained a cohort of benign prostatic hyperplasia, prostate adenocarcinoma and metastases [235]. Immunoreactivity for IGFBP-rP1 was absent from normal epithelium and BPH epithelium but was increased in prostate carcinoma and metastases. These studies also showed that prostate cancer cell lines that do not express mac25 in vitro have the ability to re-express mac25 when grown in vivo [235]. In contrast, Sprenger et al. [236], in an aggressive, SV40 T-antigen immortalized prostate cell line line, M12, recently demonstrated that overexpression of mac25 decreased cell growth and tumor formation. Therefore, the role of mac25 in differentiation and tumorigenesis will require additional study.

SUMMARY AND PERSPECTIVE

Prostate cancer research is focused on characterizing the mechanism by which prostate cancer develops initially and the process of progression of tumors from the androgen-dependent to the recurrent state. Determining the role of the IGF axis

in prostate cancer progression is an important component of this research. This chapter has described the current understanding of the IGF axis, including detailed descriptions of the IGF-1 and IGF-2 ligands, the receptors and the IGF binding proteins. Recent studies describing a strong association of elevated plasma IGF-1 with an increased risk of prostate cancer [55, 56] emphasize the critical role that IGF-1 signaling may have in prostate cancer growth. Overall, the published data support the idea that IGFs promote growth of prostate cancer cells, while the IGFBPs generally inhibit growth. The roles of cognate receptors and proteases in modulating IGF/IGFBP activity must be taken into consideration as conclusions about the IGF axis are put forth. During the development of prostate cancer the inhibitory IGFBP's (IGFBP-3, -4, and -6) are downregulated at the protein level, while the stimulatory IGFBPs (IGFBP-2 and -5) are upregulated. These changes are accompanied by increases in IGF-2 by both stromal and epithelial components. All these changes effectively increase the growth rate and survival of prostate cancer cells. Several questions can be posed to foster further exploration of the IGF axis in the prostate. Are other components of the IGF axis in the prostate yet to be discovered? Does cross-talk between growth factor signaling pathways occur? Is elevated serum IGF-1 causative for prostate cancer or do prostate tumors produce IGF-1? Is the IGF axis just one of multiple functional components leading to prostate cancer and how do these different pathways interact? Answering these questions will provide important insights into our understanding of prostate cancer development and progression and will

encourage the design of new therapeutic strategies to treat this disease.

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Table 1. Expression of IGF axis members in prostate cancer cell lines and primary cultures.

| | Cell Lines | | | | | Normal[1] | | BPH[1] | Cancer[1] |
	LNCaP	C4-2[a]	Du145	PC-3	P69[f]	Ep.[g]	Stroma[i]	Stroma	Stroma[i]
IGF-1	nd[a,c,d], +/-[b]	nd	nd[a], -[b]	-[b,c], nd[e]	-	nd	nd	+/-[h]	nd
IGF-2	+[a], +/-[b], nd[c,d]	++	nd[a], ++[b]	+[e], ++[b], nd[c]	+	nd	+	+++[h], ↑10x[i]	+
IGF1R	+[b,c], ++[a], nd[d]	+++	+[a,b]	+[b,c,e]	+	+	nd	+[h]	nd
IGF2R	+[a], ++[b], nd[c,d]	++	nd[a], ++[b]	++[b], nd[c,e]	nd	-	nd	+[h]	nd
IGFBP-1	nd[a,b,c], -[d]	nd	nd[a,b]	nd[b], -[c,e]	-	-	-	nd[h]	-
IGFBP-2	+[a,b,c], ++[d]	+	+[a,b]	+[b,d], -[c]	+	+	+	nd[h], -[i]	+
IGFBP-3	+/-[a], -[b], +[c,d]	+	+[a,b]	+[b], +/-[c], ++[e]	+	-	+	nd[h], var.[i]	+++
IGFBP-4	inc[a], +/-[b,d], -[c]	inc	inc[a], +[b]	+[b,c,e]	-	+	var.	nd[h]	var.
IGFBP-5	+++[a], -[b,d], +[c]	+/-	+[a], -[b]	+[b,c], -[e]	+	-	+	nd[h], ↑4x[i]	+
IGFBP-6	nd[a,c], -[b,d]	nd	nd[a], +[b]	++[b], -[c], +[e]	+	-	-	nd[h], -[i]	-

[a]Sikes et al., FACS, IHC, WLB, RT-PCR (unpublished results); [b]Kimura et al., RNA blot, RT-PCR IHC (J. Urol, 3:39, 1996); [c]Conover et al, WLB, WB (JCEM 80:987, 1995); [d]Goosens et al. RNA blot. (Mol. Cell. Endocrinol. 155: 9, 1999); [e]Angelloz-Nicoud and Binoux, WB, WLB, RIA (Endocronol. 136: 5485, 1995); [f]Plymate et al., RNA blot/protection (JCEM 81: 3709, 1996); [g]Cohen et al., WLB, RNA blot (JCEM 73:401, 1991); [h]Grant et al., RT-PCR (JCEM 83:3252, 1998); [i]Cohen et al., WLB, RNA blot (JCEM 79:1410, 1994)

[1]Primary Cultures from histologically normal tissues, benign prostatic hyperplasia or prostate cancer. nd = not determined; inc = inconclusive due to antibody cross-reactivity; var= variable expression

Table 2. Reported expression and localization of IGF axis members in prostate tissues.

	Prostate Tissues				Cellular Localization	
	Normal	BPH	PIN	CaP	RNA	Protein
IGF-1	nd	+[e]	nd	nd	(S>>E)	nd
IGF-2[b,d]	+	+	↑wk	↑↑30%*	(E>S)	(E=S)
IGF1R[b]	+	+	↓42%*	↓35%*	(E>>S)	(E>>S)
IGFBP-1[a]	-	-	-	-	-	nd
IGFBP-2[a,c]	+	+	+[a], ↑[c]	↑↑* (GS7-10)	+[a]	nd[a], ↑E[c]
IGFBP-3[a]	+	+	+	↓↓* (GS7-10)	+[a]	nd[a], ↑E[c]
IGFBP-4	+[a,b]	+[a,b,e,f]	nc[a],↑[b]	↑↑*[b],nc[a,f]	(E>S)[b,e]	(B+E>S wk)[b]
IGFBP-5[a,b]	+	+	↑wk	↑↑*(GS7-10)	(S**>>E)[b]	(E>B>S mod)[b]
IGFBP-6[a,b]	++	++[f]	++	+[f]	(E>S)[b]	(E>B>S wk)[b]

[a]Figueroa et al, RNase protection. (J. Urol. 159:1379, 1998.)
[b]Tennant et al, In Situ and IHC, (JCEM 81:3774 or 81:3783, 1996.)
[c]Thrasher et al, IHC, (J. Urol. 155:999, 1996.)
[d]Li et al, (Cell Tissue Res., 291:469, 1998.)
[e]Barni et al., In Situ, RIA, RNA blot (JCEM 78: 778, 1994)
[f]Goosens et al. RNA blot. (Mol. Cell. Endocrinol. 155: 9, 1999)

S=stromal localization; E=epithelial cell localization; B=basal cell localization;
wk=weak or small change; nd=not determined; GS=gleason score
*statistically significant; **strong staining in periglandular fibroblasts

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20. Feldman S.A., **Sikes R.A.**, Eckhoff G.A., Comparison of the Deduced Amino Acid Sequence of Guinea Pig Adenovirus Hexon Protein With That of Other Mastadenoviruses. <u>J. Comparative Med.</u> 51 (2): 120-126, 2001.

B. Articles in Preparation for Submission

1. Fasciana F., van der Made ACJ, **Sikes, RA**, van Weerden WM, Chung LWK, and Trapman J., A urogenital sinus cDNA (ugs148) with a specific expression pattern in embryonic and adult mouse tissues and in human prostate cancer xenografts, (Submitted <u>Cancer Res.</u> 6/00).

2. Kim J.H.,Retief J.D., Fasciana C., Wang F., Chung L., Trapman J., Chung L.W.K., and **Sikes R.A.***, The LNCaP human prostate cancer progression model differentially expresses fetal urogenital sinus-derived sequences (In preparation).   ***Corresponding Author**

3. Kao C., **Sikes R.A.**, Zhau, H.E., Li W., Huang K., Chan J., Sim R., Chang S.-M. and Chung L.W.K., p157, An osteosarcoma conditioned media-derived protein that promotes prostate cancer cell growth is human complement Factor H. (In Preparation)

4. Koeneman K.S., Yeung F., Law A., **Sikes R.A.**, Kao C, Nelson J. and Chung L.W.K., Transcriptional down regulation of PSA and enhanced growth of prostate cancer cells by osteoblastic cells., (In Preparation)

5. **Sikes R.A.***, Koeneman K.S., Edlund NM, Bissonette E.A., Nicholson B.E., Bradley MJ, Pienta K.J. amd Chung L.W.K., Cellular interactions in the tropism of prostate cancer to bone., (In Preparation) ***Corresponding Author**

6. Koeneman K.S., **Sikes R.A.**, Yeung F., and Chung L.W.K. Regulation and expression of BSP in human prostate cancer cell lines.

7. Tennant, M, **Sikes RA,** Rosenfeld R., and Plymate SR, Insulin-like growth factor binding protein-related protein 1 is reduced in human prostate cancer compared to benign prostate epithelium.

8.   Fujita-Yamaguchi Y., **Sikes RA**, Plymate SR, Expression of IGF-II during prostate cancer progression.

C. Published Articles and Book Chapters by Invitation

1.   He X-Y, **Sikes R.A.**, Thomsen S., Chung L.W.K., and Jacques S.L. Photodynamic therapy-induced programmed cell death in carcinoma cell lines. Proceedings of SPIE, 1993.

2.   Zhau H.Y.E., **Sikes R. A.**, Chung L.W.K., The c-*erb*B-2 oncogene in human prostate cancer growth and progression, pp 207-220 in:Protooncogenes and growth factors in steroid hormone -induced growth and differentiation., Ed. Khan, S.A.and Stancel, G.M., CRC press, Inc., Boca Raton, 1994.

3.   **Sikes R.A.**, Kao C and Chung L.W.K., Autocrine and Paracrine Mediators for Prostate Growth and Cancer Progression, In: Advances in Urology. Ed. E.J. McGuire, Mosby Year Books, Inc., Chicago, Volume 8, Chapter 2, pp21-60, 1995.

4.   Chung L.W.K., Kao C., **Sikes RA**, and Zhau, H.E., Human Prostate Cancer Progression Models and Therapeutic Intervention, Acta Urol. Jap., 43: 815-820, 1997.

5.   Gardner T.A., Ko S.-C., Kao C., Shirakawa T., Cheon J., Gotoh A., Wu T.T., **Sikes R.A.**, Zhau H.E., Cui Q., Balian G. and Chung L.W.K., Exploiting stromal-epithelial interaction for model development and new strategies of gene therapy for prostate cancer and osteosarcoma metastases (review), Gene Ther. Mol. Biol. 2: 41-58 , 1998.

6.   **Sikes RA**, Molecular epidemiology of cancer: the future is now., Cancer Research Alert 1(2): 13-15, 1999.

7.   Cinar B and **Sikes RA,** Control of Steroid Hormone Receptor Action., Cancer Research Alert 1(9): 100-102, 2000.

8.   Gregory CW, Degeorges A and **Sikes RA***. The role of the IGF axis in the development and progression of prostate cancer. In Recent Research Developments in Cancer, (Eds) Pandalai.S.G., Mukhtar H and Labrie F, Transworld Research Network, Kerala, India. March 2001. Volume X, Chapter Y , ppxxxx-xxxx, March 2001 *(In Press)* **Corresponding Author**

D. Abstracts Accepted for Presentation/Published (*Presented by Dr. Sikes)

1.*   **Sikes R.A.**, Petrow V., and Chung L.W.K., Effects of antiandrogen, antiestrogen, and/or 5a-reductase inhibitors on mouse prostatic hyperplasia induced by fetal urogenital sinus implant., Organ Systems Program Workshop, Stromal-Epithelial Interactions, April 16-18, Rockville, MD, 1986.

2.*   **Sikes R. A.**, and Chung, L.W.K., Transformation of rat prostatic epithelial cells with activated *myc* and *neu* oncogenes., American Urologic Association, Inc., Podium Session (AUA) 84th   Annual Meeting, Dallas, TX, J. Urol. 141: 218A, 1989.

3.*   **Sikes R.A.**, and Chung L.W.K., Transformation or rat prostatic epithelial cells by overexpression of an activated *neu* oncogene.,American Urologic Association, Inc., (AUA) 85th Annual Meeting, New Orleans, LA, J. Urol., 143: 228A, 1990.

4.*   **Sikes R. A.**, and Chung L.W.K., *Neu* oncogene associated changes in gene expression: correlation with *neu* overexpression and tumorigenicity in rat ventral prostate epithelial cells., Podium Session, Society for Basic Urologic Research, Fall Symposium, Rochester, MN, 1991.

5.   Wu H.-C., Hsieh J.-T., Gleave M.E., **Sikes R. A.**, Logothetis C.J., and Chung L.W.K., Combination therapy with pseudomonas exotoxin fusion protein (TP40) and retinoic acid in human transitional cell carcinoma ell lines., American Urologic Association, Inc., (AUA) 87th Annual Meeting, Washington, D. C., J. Urol., 147: 404A, 1992.

6.* **Sikes R.A.** and Chung L.W.K., Neu associated changes in gene expression: correlation with neu overexpression and tumorigenicity in rat prostate epithelial cells., 1992 International Symposium on Biology of Prostate Growth, Bethesda, MD, 1992.

7. Zhou J., **Sikes R.A.**, and Zhau H.Y.E., The role of *neu* oncogene in prostatic epithelial carcinogenesis., Society for European Urologic Research, Trento, Italy, 1992.

8. He X-Y, **Sikes R.A.**, Chung L.W.K., and Jacques S.L., Photodynamic therapy induced programmed cell death of prostatic cells: a comparison with the result in breast and lung cancer cells. American Urological Association (AUA) Annual Meeting, San Antonio, TX, 1993.

9. He X-Y, **Sikes R.A.**, Thomsen S., Chung L.W.K., and Jacques S.L., Photodynamic therapy-induced programmed cell death in carcinoma cell lines. Proceedings of the SPIE, Los Angeles, CA, 1993.

10. Zhou J., **Sikes R.A.**, and Zhau H.Y.E., Transfection of mutated c-erb B2/*neu* oncogene induces human prostate cancer metastasis., American Urological Association (AUA) Annual Meeting, San Antonio, TX, J. Urol 149(4):374A, 1993.

11. Thalmann G.N., Hall M.C., **Sikes R.A.**, Devoll R.E., Farach-Carson M.C., von Eschenbach A.C., and Chung L.W.K., Role of bone matrix proteins vitronectin, osteopontin, and alpha-2HS glycoprotein on prostate cancer progression and osseous mateastasis., European Society for Urological Oncology and Endocrinology 10th Congress, Berne, Switzerland, March 10-13, 1994.

12. Thalmann G.N., **Sikes R.A.**, Hall M.C., Devoll R.E., Farach-Carson M.C., von Eschenbach A.C., and Chung L.W.K., Implications of bone matrix protein osteopontin (OPN) on prostate cancer progression and osseous metastasis, Society for Basic Urologic Research (SBUR), San Francisco, CA, May 13-14, 1994.

13.* **Sikes R.A.**, Kao C. and Chung L.W.K., p157, An osteosarcoma conditioned media-derived protein that promotes prostate cancer cell growth, is human complement factor H., AACR special conference: Basic and Clinical Aspects of Prostate Cancer, Palm Springs, CA Dec. 8-12, 1994.

14. Thalmann G.N., **Sikes R.A.**, Chang S.M., Pathak S., von Eschenbach A.C. , and Chung L.W.K.., The LNCaP model of human prostate cancer: Biological behaviours and cytogenetic changes., Urol. Res., 23(4): 261, 1995.

15. Thalmann G.N., **R.A. Sikes**, R.E. Devoll, S. Pathak, M.C. Farach-Carson, A.C. von Eschenbach, and L.W.K. Chung, Androgen-independent and osseous metastatic progression of human prostate cancer: Model development and roles of osteopontin., J. Urol. 153 (4): 312A, 1995.

16. Thalmann G.N., **Sikes R.A.**, Chang S-M.C., Johnston D.A., von Eschenbach A.C. and Chung L.W.K., Suramin down regulates prostate-specific antigen (PSA) expression in androgen-dependent LNCaP and androgen-independent C4-2 human prostate cancer cell lines., American Urological Association (AUA) Annual Meeting, Las Vegas, NV 1995. J. Urol. 153 (4): 269A, 1995.

17. Chen M.E., Shen J.C., Thalmann, G.N., **Sikes R.A.**, von Eschenbach A.C. and Chung, L.W.K., Androgen-independent human prostate cancer progression: the isolation of novel stage-specific sequences using differential mRNA display., American Urological Association (AUA) Annual Meeting, Las Vegas, NV 1995. J. Urol. 153 (4): 267A, 1995.

18. Thalmann G.N., **Sikes R.A.**, Devoll R.E., Farach-Carson C.M., and Chung. L.W.K., Osteopontin (OPN): Para- und autokriner Mediator beim Prostatakarzinom.,Urologe A, Suppl. 1, 35: S147, 1996.

19. Chen M.E., **Sikes R.A.**, Wu W.G., Troncoso P., Lin S.H., von Eschenbach A.C., and Chung, L.W.K., PAGE-1 and GAGE-7 are novel genes expressed in the LNCaP prostatic carcinogenesis model that share homology with melanoma associated antigens., American Urological Association (AUA) Annual Meeting, Orlando, FL 1996.

20. Thalmann G.N., Troncoso P., Devoll R.E., **Sikes R.A.**, Ball R., Studer U.E., and Chung L.W.K., Osteopontin (OPN) in prostate cancer: immunohistichemical and molecular analysis of expression and distribution. American Urological Association (AUA) Annual Meeting, New Orleans, LA 1997, J. Urol., 157 (Suppl.): 81A, 1997

21. Thalmann G.N., Troncoso P., Devoll R.E., **Sikes R.A.**, Ball R., Studer U.E., Farach-Carson M.C., and Chung L.W.K., Osteopontin (OPN) in prostate cancer: Biological activity, immunohistochemical and molecular analysis of expression and distribution., Urol. Res., 25(1): 77, O2.3, 1997.

22. Thalmann G.N., **Sikes R.A.**, Chang S.M., Ozen M., Pathak S., and Chung L.W.K., The LNCaP mouse model of human prostate cancer (Pca): androgen-independence and osseous metastasis., Urol. Res. 25(1): 79, O2.14, 1997.

23. Miyamoto T., Wu T.T., Li H., Kao C., **Sikes R.A.**, Zhau H.E., and Chung L.W.K., Prostate growth and PSA expression are affected by stroma-epithelial interaction in collagen gel: stroma cell specificity and the roles of growth fqactors and extracellular matrices. American Urological Association (AUA) Annual Meeting, New Orleans, LA 1997, J. Urol., 157 (Suppl.):

24. Degeorges A., Wang F., Frierson Jr H.F., Seth A., **Sikes R.A.**, Expression of IGFBP-7/mac25 in prostate cancer. Proceedings of the American Association for Cancer Research (AACR) Vol 39, pg 110 # 748 1998 New Orleans, LA.

25. Degeorges A., Wang F., Frierson Jr H.F., Seth A., **Sikes R.A.**, Expression of IGFBP-7/mac25 in prostate cancer. International Symposium on Biology of Prostate Growth, NIH Bethesda MD, 1998

26.* **Sikes R.A.**, Kim J.-H., Wang F., Degeorges A., Chung, L.W.K., Fasciana C., Trapman J., Novel fetal urogential sinus gene expression in prostate cancer progression. International Symposium on Biology of Prostate Growth, NIH Bethesda MD, 1998

27. Theodorescu D, Frierson HF, Mills SE, **Sikes RA**, Charlottesville , VA , Molecular determination of surgical margins following radical prostatectomy, American Urological Association (AUA) Annual Meeting, San Diego, CA 1998

28. Koeneman KS ; Yeung F; **Sikes RA;** Chung LWK ; Charlottesville, VA; Nelson JB ; Baltimore, MD Transcriptional down regulation of PSA, and enhanced growth of prostate cancer cells (CaP) by co-culture with osteoblastic cells, American Urological Association (AUA) Annual Meeting, Dallas TX 1999

29.* **Sikes RA***, Koeneman KS. , Bissonnette EA , Pienta KJ and LWK Chung, Cellular Interactions in the Tropism of Prostate Cancer to Bone. Cancer Research at the Millennium. 42nd Annual Clinical Conference, 52nd Annual Symposium on Fundamental Cancer Research, Houston TX Jan 9-12, 2000.

30. Jung CJ, Ou Y-C, Ko SC, **Sikes RA**, Zhau HE, Chung LWK, Diferentiation in level of human osteocalcin expression between primary and metastatic prostate tumors. Proceedings of the AACR 41: #258, 91st Annual Meeting of the AACR, SanFrancisco CA April 1-5, 2000

31. Koeneman KS, **Sikes RA**, Yeung F, Hsieh C-L ,Chung LWK, Sodek J, Adenovirus mediated gene therapy can target metastatic prostate cancer by using the bone sialoprotein (BSP) promoter., American Urological Association (AUA) Annual Meeting, Atlanta GA 2000.

RESEARCH INTERESTS:

1. The IGF axis is comprised of six high affinity binding proteins, IGFBPs 1 to 6, and two high affinity receptors, IGF1R and IGF2R. The IGF1R is a heterodimeric receptor tyrosine kinase (RTK) and has no other known ligands besides IGF-I and IGF-II. The IGF2R has no known signaling function and it binds both IGF-II and mannose-6-phosphate with high affinity. The IGF2R has a reduced affinity for IGF-I, Alterations in the IGF axis, namely changes in expression levels of various IGFBPs and the IGF receptors, have been implicated in the initiation, development and progression of both BPH and PCa. Research in my laboratory is trying to determine the interplay between the IGF axis and the androgen receptor with particular emphasis on changes that occur during progression of PCa to an androgen independent phenotype. In addition to testing the signaling via the IGF1R, I am testing the responsiveness of the various IGFBPs to androgen in a progression model of human prostate cancer. The aim of this research is to elucidate the mechanism(s) of prostate cell survival and growth during progression to androgen independence. Identification of the components of this system should allow for the development of therapeutic compounds directed at specific molecules involved in the process. Current approaches include the use of retrovirally delivered ER-localized scFv to the IGF1R and microarray screening to identify candidate genes in the prostatome regulated by both IGFI and androgen. Additional studies are characterizing the role of the IGFBP's in prostate development.

2. Analysis of novel gene expression during prostate gland development and the application of these findings to BPH and malignancy or **Onco-fetal translational genomics**. We have cloned the expressed RNAs of mouse urogenital sinus (E15.5-16.5) into bacterial libraries. This project involves: 1) The expansion and direct sequencing of these expressed sequence tags (ESTs) and comparison to international sequence databases to ascertain the uniqueness of each sequence. To date the project has yielded about 52% unique sequences from the 787 ESTs sequenced to date as compared to the GenBank database using FASTA analysis. 2) The unique clones will be narrowed using biochemical and sequence localization techniques to ascertain those with relevance to prostate biology, i.e. prostate development or disease progression. To this end a matrix array of the clones was hybridized to both LNCaP, androgen sensitive PCa, or C4-2, androgen independent PCa, radiolabeled single-stranded cDNAs. This approach has yielded 31 fetal sequences whose expression level changes from LNCaP to C4-2 indicating a potential association with PCa progression. 3) I am now in the process of cloning the full-length open reading frames for some of the ESTs with the largest changes in expression level.

   The examination of the UGS library will be expanded to include an examination of the directed expressionof UGS RNAs in a lobe and temporal specific manner. The goal is to determine which UGS genes represent a core of genes required for the development and maintenance of the different lobes of the prostate. The data will then be examined for their applicability as markers for human prostate tissue. Some of these may have utility in prostate cancer diagnosis, prognosis and/or therapeutic application.

3. Stromal-epithelial interaction: emphasis towards prostate cancer metastasis to bone and the unique behavior/biochemistry of bone-prostate interaction. We are currently evaluating this interaction by : 1) developing new techniques to study prostate cancer: bone interaction using the androgen dependent (LNCaP) or androgen independent, LNCaP-derived (C4-2) cell lines. Using these new models we are able to screen new compounds for their efficacy in androgen-dependent and -independent prostate cancer. 2) discovering novel gene sequences associated with prostate cancer progression. By ascertaining the pattern and cell specificity of these genes' expression we hope to gain insight into their function and role in prostate cancer progression and metastasis. Growth, differentiation and signal transduction will be the focus of study for these genes

4. Differential expression techniques have been employed successfully to clone and characterize novel progression associated genes in the LNCaP human prostate cancer progression model. These genes, PAGE-1 and GAGE-7, have homology to the GAGE-family of melanoma antigenic epitope. The function of these molecules is currently unknown, however, they are often re-expressed in cancer tissues and have been found to produce autologous reactive CTL clones from patient PBLs. The MAGE-family of more distantly related molecules has shown utility in melanoma patients as the origin of novel peptide-based vaccines. Therefore, PAGE-1 and GAGE-7 are being studied further to examine their role in prostate cancer progression as well as to determine their utility for development as prostate cancer therapy.

PERSONAL REFERENCES:

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PATENTS (Pending & Provisional)

1. Utilization of osteocalcin promoter to deliver therapeutic genes to osteosarcoma, glioma, melanoma, lung and prostate cancers and their osseous metastases. Chung LWK, Kao C, Ko S-C, Cheon J, and **Sikes RA** (US Patent # 5,772,993).
2 In vivo suppression of osteosarcoma pulmonary metastasis with intravenous osteocalcin promoter-based toxic gene therapy. Chung LWK, Kao C, **Sikes RA,** Ko S-C, Cheon J, and Sung BKS, (US Patent # 6,159,467).
3.. Isolation and use of fetal urogenital sinus expressed sequences. **Sikes RA**, Kim JH, Fasciana C, Trapman J, and Chung LWK (Provisional #60/085,383 5/14/98, Patent Conversion initiated June 2000).
4. Detection of residual cancer during radical prostatectomy. Theodorescu D and **Sikes RA** (Provisional #60/095,484 8/13/98)
5. PAGE-1, a prostate cancer associated gene with immunotherapeutic potential. **Sikes RA**, Chen ME, Lin S-H, and Chung LWK, (Provisional Submitted)

ADMINISTRATIVE RESPONSIBILITIES

I. Teaching

  A. Research Oriented

    1. Participation or Direction in the training of Clinical Research Fellows (1987-present):

      a. Louis Pisters, M.D. (AFUD scholar) 1991-1993
        Current Position: Asst Professor of Urology, MD Anderson Cancer Center, Houston TX
      b. George N. Thalmann, M.D. 1993-1995
        Current position: Assoc. Professor of Urology, University of Bern, Bern, Switzerland
      c. Michael E. Chen, M.D. (AFUD scholar) 1993-1995
        Current Position: Asst Professor of Urology, MD Anderson Cancer Center, Houston TX
      d. Tony Wu, M.D. 1995-1997
        Current Position: Asst. Professor of Urology, Veterans General Hospital-Kaohsiung,Taiwan

e. Mitchell H. Sokoloff, M.D. (AFUD scholar) 1997-1999
   Current Position: Asst. Professor of Urology, University of Chicago, Chicago IL
f. Kenneth K. Koeneman, M.D. (AFUD scholar) 1998-2000
   Current Position: Asst. Professor of Urology, U.T. Southwestern, Dallas TX
g. Brian E. Nicholson, M.D. 2000-current (Urology Training Grant)
   Current Position: Research Instructor, Dept. of Urology, University of Virginia, Charlottesville VA

2. Urology Resident Research Training:

Jin-Hee Kim, M.D. 1997-1998
Current Position: Urology Resident UCLA Medical School

3. Post-doctoral Fellows:

a. Armelle Degeorges, Ph. D. 1996-1998
   Current Position: Research Scientist at Neurotech S. A., a Genopole Industries
b. Chaeyong Jung, DVM, PhD 1998-2000
   Current Position: Research Assistant Professor, Indiana University Department of Urology

4. Independent Study Undergraduates : Biology 495-496. Independent Study Mentor

a. Nila Rafiq, UVa 3rd year, Biology Major
        Term: September 1996-May 1997
b. Thomas-Pierre Phillippe, UVa 3-4th year, French Major
        Term: January 1997-December 1997
c. Dannon Smith, UVa 4th year, Biology Major
        Term: Sept. 1999-May 2000

B. Invited Lectureships and Courses

1. Interim Lecturer (Anatomy and Physiology), Houston Community College, Houston, Texas 1994.
2. Group presentation on, "Prostate cancer progression associated genes." UVa Mini-Medical School 1996.
3. University of Virginia Matrix Group, "Role of Osteopontin in Prostate Cancer Progression." April 1997.
4. IBC's International Symposium on Prostate Cancer: Advances in Diagnostics and Therapeutic Development. Amelia Island Plantation, FL "Isolation and Characterization of Novel Genes Associated with Prostate Cancer Progression" November 1998 and session chair.
5. Centre Nationale de la Recherche Scientifique, Paris ,France, December 10, 1999. "Regulation of IGF Binding Protein Expression in the LNCaP Model of Human Prostate Cancer progression"
6. Department of Urology, University of Bern, Bern, Switzerland December 13-14, 1999.
    a) "Regulation of IGF Binding Protein Expression in the LNCaP Model of Human Prostate Cancer progression"
    b) "Expression Pattern of IGFBP-rP1 in Normal Human Tissues and Prostate Cancer"
7. Feist-Weiler Cancer Center, LSU Medical Center, Shreveport LA, March 5, 2001.   "The IGF axis in prostate cancer progression."
8. Department of Biological Sciences, University of Delaware, Newark DE, May 4, 2001. "The IGF axis in prostate cancer progression."
9. Karmanos Cancer Center and Department of Urology, Wayne State University, Detroit MI, May 9, 2001. "The IGF axis in prostate cancer progression."
10. Man to Man of the Valley, Prostate Cancer Support Group. May 17, 2001, Augusta Medical Center, Fishersville, VA. " The role of insulin-like growth factors in the development and progression of prostate cancer."

II. <u>Peer-Review Process</u>

A. Manuscript reviews (Ad hoc basis).

1. Biology of Reproduction
2. Cancer Research
3. Developmental Dynamics
4. Endocrinology
5. J. Investigative Urology
6. Journal of Biological Chemistry
7. Molecular Carcinogenesis
8. The Prostate
9. Urology
10. British Journal of Cancer

B. Non-NIH Grants Reviewed:

1. Department of Veterans Affairs Extramural Review Program _____(3)
2. MD Anderson Extramural Review of Institutional Grants _____(1)
3. University Grants Committee-Hong Kong _____(3)

C. Study Sections (NIH and DOD)

1. Ad Hoc Member - Program Project Site Visit: PO1 CA84203-01; Hasan, T- PI Boston, MA June 2-4, 1999
2. Ad Hoc Member - NCI Scientific Review Group, Subcommittee C, Program Project Parent Committee, Washington DC, August 10-12, 1999.
3. Full Panel Member- Department of Defense, USAMRMC Prostate Cancer Research Program FY2000, Washington DC, July 6-8, 2000.
4. Full Panel Member- Department of Defense, USAMRMC Prostate Cancer Research Program FY2001, Washington DC, May 20-22, 2001.

III. <u>Editorial Board(s)</u>

Cancer Research Alert. (American Health Consultants)
       Editor-in-Chief: David Corral, M.D.
       Monthly Serial Publication-Initiated 1999-present